Profiling Phosphoproteins of Yeast Mitochondria Reveals a Role of Phosphorylation in Assembly of the ATP Synthase*[S]

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Mitochondria are crucial for numerous cellular processes, yet the regulation of mitochondrial functions is only understood in part. Recent studies indicated that the number of mitochondrial phosphoproteins is higher than expected; however, the effect of reversible phosphorylation on mitochondrial structure and function has only been defined in a few cases. It is thus crucial to determine authentic protein phosphorylation sites from highly purified mitochondria in a genetically tractable organism. The yeast *Saccharomyces cerevisiae* is a major model organism for the analysis of mitochondrial functions. We isolated highly pure yeast mitochondria and performed a systematic analysis of phosphorylation sites by a combination of different enrichment strategies and mass spectrometry. We identified 80 phosphorylation sites in 48 different proteins. These mitochondrial phosphoproteins are involved in critical mitochondrial functions, including energy metabolism, protein biogenesis, fatty acid metabolism, metabolite transport, and redox regulation. By combining yeast genetics and *in vitro* biochemical analysis, we found that phosphorylation of a serine residue in subunit g (Atp20) regulates dimerization of the mitochondrial ATP synthase. The authentic phosphoproteome of yeast mitochondria will represent a rich source to uncover novel roles of reversible protein phosphorylation. *Molecular & Cellular Proteomics 6:1896–1906, 2007.*

Mitochondria are the central organelle for the energy metabolism of eukaryotic cells and are critical for numerous metabolic pathways, including that for amino acids, lipids, heme, and iron-sulfur clusters, and play key roles in the regulation of programmed cell death (1–5). It is evident that these processes have to be tightly regulated to permit a mitochondrial response to changes in energy demand, cellular metabolism, or environmental conditions (6, 7). Until recently the most common regulatory mechanism of eukaryotic cells, reversible phosphorylation (8–10), was considered to represent an exception in the case of mitochondria, including the E1 subunit of pyruvate dehydrogenase and the branched-chain α-ketoacid dehydrogenase (11–14).

A number of recent studies have provided evidence that phosphorylation of mitochondrial proteins is much more frequent than expected (15–23). (i) Incubation of isolated mitochondria with radiolabeled ATP or staining of mitochondrial proteins with phosphospecific dyes suggested that a substantial fraction of mitochondrial proteins are phosphorylated (24–27). A limitation of these approaches is the inability to identify the specific phosphorylated amino acid residues in addition to the possibility of nonspecific labeling of proteins. (ii) Proteomics analysis of isolated mitochondria by mass spectrometry revealed the presence of numerous protein kinases and phosphatases (28–34), implying that reversible protein phosphorylation may be a widespread mechanism of regulating mitochondrial function. The most comprehensive proteomics analysis of mitochondria, the PROMITO study of highly purified *Saccharomyces cerevisiae* mitochondria, led to the identification of ~850 different proteins and a coverage of ~85% of the known mitochondrial proteins (28, 34). The PROMITO dataset includes more than a dozen predicted protein kinases/phosphatases. Two of them, encoded by the open reading frames YIL042c and YOR090c, were indeed subsequently shown to function as the kinase/phosphatase system that regulates reversible phosphorylation of the yeast pyruvate dehydrogenase complex in the mitochondrial matrix (35). (iii) Mass spectrometry is the most efficient method to identify the exact phosphorylation sites within proteins/peptides. A variety of proteomics techniques can be applied from specific enrichment of phosphorylated proteins/peptides to phosphospecific scanning techniques at the MS level. Lee *et al.* (36) identified phosphorylated proteins in the mitochondrial fraction from mouse liver. Ficarro *et al.* (37), Gruhler *et al.* (38),

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and Chi *et al.* (39) analyzed the phosphoproteome of whole yeast cell extracts. Among several hundred phosphoproteins they identified only a few mitochondrial proteins, suggesting that the studies were below saturation level at least for mitochondrial proteins. A limitation of many proteomics studies is the purity of the mitochondrial preparation, and due to contamination of mitochondria with various amounts of other cellular fractions, the assignment of newly identified phosphoproteins to mitochondria can be ambiguous.

S. cerevisiae is a major model organism for the characterization of mitochondrial functions due to its unparalleled genetic and biochemical tractability. The functions of many mitochondrial proteins, *e.g.* of chaperones, receptors, translocators, and assembly factors, have been identified in yeast first. Moreover yeast is of increasing importance in defining mitochondrial functions in programmed cell death (40–42) and for characterization of the molecular basis of human mitochondrial diseases (43–47). Together with the development of an efficient protocol for the isolation of highly pure mitochondria, yeast is an optimal organism for a comprehensive proteomics and functional analysis of mitochondria (28, 33, 34, 48, 49). The authentic phosphoproteome of pure yeast mitochondria has not been reported so far. Here we performed a profiling of the phosphorylation sites of yeast mitochondrial proteins by a global approach and a phosphospecific approach with the latter focusing on different IMAC systems as well as on strong cation exchange chromatography for the enrichment of phosphorylated peptides. We identified 48 phosphoproteins and defined 80 phosphorylation sites. Among the newly identified phosphoproteins was subunit g (Atp20) of the mitochondrial ATP synthase (F_0F_1-ATPase),[1] the machinery in the mitochondrial inner membrane and matrix that is responsible for ATP production. By combining yeast genetics and native gel electrophoresis, we demonstrate that phosphorylation of serine 62 of Atp20 is critical for regulating assembly of the ATP synthase.

EXPERIMENTAL PROCEDURES

Isolation of Yeast Mitochondria—The wild-type yeast strain YPH499 was grown on non-fermentable medium (YPG: 1% (w/v) yeast extract, 2% (w/v) bactopeptone, and 3% (w/v) glycerol) at 30 °C to an OD of approximately 1. Cells were homogenized in the presence of phosphatase inhibitor mixtures I and II (Sigma-Aldrich). Crude mitochondria were isolated by differential centrifugation and further purified via a three-step sucrose gradient as described previously (28, 34, 48, 49). Highly purified mitochondria were resuspended in SE buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2) and stored in aliquots at −80 °C.

IMAC Enrichment after SDS-PAGE—Highly purified mitochondria (400 μg of protein) were separated on a BisTris SDS-PAGE system (NuPAGE, Invitrogen). After visualization by colloidal Coomassie staining (50), gel lanes were cut into 1-mm broad slices, which were subjected to in-gel digestion according to Shevchenko *et al.* (51) with slight modifications. Vacuum-dried gel pieces were incubated with 12.5 ng/μl trypsin (sequencing grade modified, Promega, Madison, WI) in 50 mM NH_4HCO_3, pH 7.8, at 37 °C overnight. Peptides were extracted twice with 10 μl of 5% formic acid (FA) and once with 10 μl of 5% FA, 50% ACN. For each gel lane extracts were combined, concentrated under vacuum, and further enriched for phosphopeptides by IMAC (37). Subsequently 10 μl of Phos-Select™ Iron Affinity Gel (Sigma-Aldrich) was washed twice with loading buffer (250 mM acetic acid, 30% ACN) and combined with the peptide extract, and volumes were adjusted to 100 μl with loading buffer. After incubation for 1 h at room temperature, Phos-Select beads were washed three times with loading buffer and once with ultrapure water, and phosphopeptides were eluted with 30 μl of 400 mM NH_4OH, pH 10.5. Eluates were acidified with TFA and analyzed by nano-LC-MS/MS.

One-step IMAC in Solution—Highly purified mitochondria (300 μg of protein) were lysed in 50 mM Tris, pH 7.8, 150 mM NaCl, 0.5% (w/v) SDS, phosphatase inhibitor mixtures I and II (Sigma-Aldrich) and digested in-solution with 6 μg of trypsin in 50 mM NH_4HCO_3, pH 7.8, at 37 °C overnight. IMAC was carried out as described above but with larger volumes and prolonged steps. The sample was diluted 1:10 with loading buffer and incubated with 80 μl of bead slurry for 4 h. Washing and elution were accomplished in SigmaPrep™ Spin Columns (Sigma-Aldrich) according to the manufacturer's recommendations. Similarly Ga^{3+}- and Zr^{4+}-coated Poros™ MC20 (PerSeptive Biosystems, Foster City, CA) beads were used, leaving out the washing step with water.

Two-step IMAC in Solution—Mitochondrial lysate (500 μg of protein) was diluted 1:10 with loading buffer, and IMAC was carried out at the protein level as described before for the one-step procedure. However, instead of acidifying with TFA, eluates were brought to pH 7.8 with 50 mM NH_4HCO_3 and subjected to overnight digestion at 37 °C using 10 μg of trypsin. Afterward proteolytic digests were enriched for phosphopeptides as described above.

Strong Cation Exchange (SCX) Enrichment of Phosphopeptides—A tryptic digest of highly purified mitochondria (500 μg of protein) was diluted 5-fold with 5 mM NaH_2PO_4, pH 2.7. Phosphopeptides were enriched according to Beausoleil *et al.* (52) with slight modifications. A 2.1-mm-inner diameter × 15-cm-long column (PolySULFOETHYL Aspartamide, 200-Å pore size, 5-μm particle size, Chromatographic Technologies, Basel, Switzerland) was used in combination with an Ultimate™ HPLC system (Dionex, Idstein, Germany). Chromatographic separation was accomplished using a binary buffer system consisting of 5 mM NaH_2PO_4, pH 2.7 (buffer A), and 5 mM NaH_2PO_4, 15% acetonitrile, 500 mM NaCl, pH 2.7 (buffer B), at a flow rate of 150 μl/min. Phosphopeptide-enriched fractions were collected every minute with a ProteineerFC fraction collector (Bruker Daltonics, Bremen, Germany).

MS Acquisition—Nano-LC-MS/MS analyses were performed on a Qstar®XL, a Qtrap™ 4000 (both Applied Biosystems, Darmstadt, Germany), and an LCQ Deca XPplus™ (Thermo Electron, Dreieich, Germany), respectively, coupled to Ultimate or Ultimate 3000 nano-HPLC systems (Dionex). The HPLC systems were configured in a preconcentration setup with a 150-μm-inner diameter reverse phase trapping column (53). Peptides were separated on a 75-μm-inner diameter reverse phase main column by applying a 40-min binary gradient (solvent A: 0.1% FA; solvent B: 0.1% FA, 84% ACN) ranging from 5 to 95% solvent B at a flow rate of 270 nl/min. Full MS scans from 400 to 2000 *m/z* (LCQ Deca XPplus) and from 350 to 2000 *m/z* (QstarXL and Qtrap 4000) were recorded, and the four (LCQ Deca XPplus and Qtrap 4000) or two (QstarXL) most intensive peaks were subjected to MS/MS taking into account a dynamic exclusion.

[1] The abbreviations used are: F_0F_1-ATPase, mitochondrial ATP synthase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; FA, formic acid; SCX, strong cation exchange; MRM, multiple reaction monitoring; MAP, mitogen-activated protein; TOM, translocase of the outer membrane.

MS Data Interpretation—MS data were transformed into mgf format using the following software and respective parameters. For the Qtrap 4000, Analyst 1.4 with the mascot.dll plug-in 1.6b5 (Matrix Science) was used. Charge states were determined from enhanced resolution scans. Spectra with less than 10 ions were omitted. Peaks with intensities 0.1% below base peak were removed, and data were centroided. For the QstarXL, Analyst 1.1 QS with the mascot.dll plug-in 1.6b7 was used. Charge states were determined from survey scans. Spectra with less than 10 signals were omitted, precursor mass tolerance for grouping was set to 0.05 Da, and maximum number of cycles between groups was set to 3. Peaks with intensities 0.1% below base peak were removed. For the LCQ Deca XPplus, LCQ_dta.exe (December 2005) on the basis of Xcalibur 1.4 and 2.0 format as plug-in to the Mascot™ Daemon version 2.1.6 was used. Precursor charge was set to auto, and grouping tolerance was set to 1.4 Da. Minimum scans per group and intermediate scans were set to 1. Generated peak lists were searched against the *Saccharomyces* Genome Database (SGD) (www.yeastgenome.org, 6712 sequences, November 2005) using Mascot version 2.1.03 (54).

Carbamidomethylation of Cys residues (if accomplished) as fixed modification and phosphorylation of Ser/Thr/Tyr as variable modification were taken into account. Moreover database searches were repeated with acetylation of protein amino termini and oxidation of methionine as additional variable modifications. Trypsin with a maximum of two missing cleavage sites was chosen as enzyme, and peptide and MS/MS tolerances in Da were selected depending on the respective mass analyzers (0.2/0.5 for the QstarXL, 0.4/0.4 for the Qtrap 4000, and 1.5/1.5 for the LCQ Deca XPplus).

Only phosphopeptide spectra with a minimum Mascot score of 32 ($p < 0.05$ or below for all utilized mass analyzers) were considered for further manual data interpretation. Moreover all spectra were validated and verified manually using MS-Product (University of California San Francisco Mass Spectrometry Facility) with loss of H_3PO_4 and multiple losses enabled as additional option, focusing on the occurrence of b- and y-ions, internal fragments, and neutral losses of H_2O, NH_3, or H_3PO_4 from these ions and the parent ion, respectively. Phosphorylation site assignment by Mascot was again verified manually. Furthermore all phosphopeptides were inspected for redundancy and represent sequences that are unique within the utilized database. Peptides for which phosphorylation sites could not be assigned unambiguously are labeled in the respective tables.

Multiple Reaction Monitoring—To determine appropriate transitions for multiple reaction monitoring (MRM) one dominant transition for each previously identified phosphopeptide was defined from the acquired mass spectra. The respective MRM transitions were analyzed afterward on the Qtrap 4000 ESI-MS system described before. 5 μg of tryptic mitochondrial digest were preconcentrated on a 3-cm trapping column (ACE C_{18}, 100-μm inner diameter, 5-μm particle size, 100-Å pore size, Hichrom Ltd., Berkshire, UK) and separated using a 4-h gradient at a flow rate of 290 nl/min ranging from 5 to 45% solvent B on a 30-cm main column (ACE C_{18}, 75-μm inner diameter, 3-μm particle size, 100-Å pore size) heated to 60 °C with an in-house built column oven. Q1 and Q3 resolutions were set to high and unit, respectively. A total of 80 MRM transitions (20 ms each) were used to trigger enhanced resolution scans and subsequent enhanced product ion scans of the two most abundant ions. Three mitochondrial phosphopeptides were identified reproducibly in the complete tryptic digest of mitochondrial lysate by nano-LC-MS/MS (without prior enrichment of phosphopeptides): two peptides from Zeo1 (K ↓ NEApTPEAEQVK ↓ K and K ↓ EQAEApSIDNLK ↓ N where pT is phosphothreonine and pS is phosphoserine) and one from Pda1 (R ↓ YGGHpSMSDPGTTYR ↓ T). Identified phosphopeptides were selected to set up a pure MRM acquisition method. Therefore, six transitions per phosphorylated and non-phosphorylated isoform of each peptide ($6 \times 3 \times 2$) were defined and monitored for 20 ms each. Q1 and Q3 resolutions were both set to high. Only peak areas where all six corresponding MRM transitions overlapped were considered as specific, and the respective peak areas were summed. Determined signal intensities were used for an approximate assessment of the degree of phosphorylation as Steen *et al.* (55) have shown that 70% of phospho/non-phospho pairs show ionization/detection efficiencies in the range of 0.5–2.0 where a number >1 indicates a better ionization/detection efficiency of the non-phosphorylated peptide. The peptide K ↓ NEApTPEAEQVK ↓ K from Zeo1 generated distinct peaks for the six transitions of each of the non-phosphorylated and phosphorylated sequences. The peak areas for all six related MRM transitions were summed to 1.3×10^6 counts for the phosphorylated and 1.6×10^7 counts for the non-phosphorylated peptide, leading to an estimated phosphorylation ratio of 4–17% (determined from intensity ratio of 12.3 × ionization/detection efficiency of 0.5–2.0). The peptide K ↓ EQAEApSIDNLK ↓ N from Zeo1 yielded MRM results that could not be interpreted with significance. The Pda1 peptide R ↓ YGGHpSMSDPGTTYR ↓ T was only detected in the phosphorylated form, whereas the non-phosphorylated isoform was reproducibly not detectable neither by MRM scanning nor by inclusion list scanning on a Qstar Elite nano-LC-MS/MS system (doubly and triply charged forms), suggesting a high degree of phosphorylation of this peptide.

Mutagenesis of ATP20—The ORF of yeast *ATP20* (subunit g) including an additional 300 bp at both the 5′- and 3′-ends of the ORF was amplified by PCR and cloned into the pRS413 vector containing a *HIS3* marker gene (56). The point mutations $ATP20^{S62E}$ and $ATP20^{S62A}$ were introduced with a QuikChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The plasmids were transformed into an *atp20*Δ strain (Euroscarf accession number Y05439), and the cells were grown on selective medium minus histidine.

In Vitro Protein Import—Radiolabeling of preproteins with [^{35}S]methionine was performed in an *in vitro* transcription/translation system in reticulocyte lysate as described before (57, 58). Import reactions into isolated mitochondria were performed as described previously and analyzed by digital autoradiography (57, 58).

Blue Native Electrophoresis and Western Blotting—Mitochondria were solubilized in digitonin-containing buffer (1% digitonin, 20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM PMSF) and separated on a 5–10% gradient blue native gel (59, 60). After electrophoresis, proteins were transferred to a PVDF membrane. Proteins of interest were visualized with the appropriate antibodies, a horseradish peroxidase-coupled secondary antibody, and the ECL detection system (GE Healthcare) (60).

RESULTS

Identification of Phosphorylation Sites of Yeast Mitochondrial Proteins—S. cerevisiae mitochondria of very high purity were obtained by differential centrifugation and subsequent sucrose gradient centrifugation (28, 34, 48, 49). We used several parallel approaches to maximize the number of identified phosphorylation sites (Fig. 1*A* and Supplemental Table 1). Phosphorylated species were enriched at the protein and/or peptide level using IMAC as well as SCX and analyzed by nano-LC-MS/MS. Additionally lysed mitochondria were separated by SDS-PAGE. The gel was cut into small slices, subjected to in-gel digestion, and analyzed either directly by nano-LC-MS/MS or after enrichment of phosphopeptides by IMAC. Database searches were undertaken using Mascot, and all phosphopeptide spectra were manually validated.

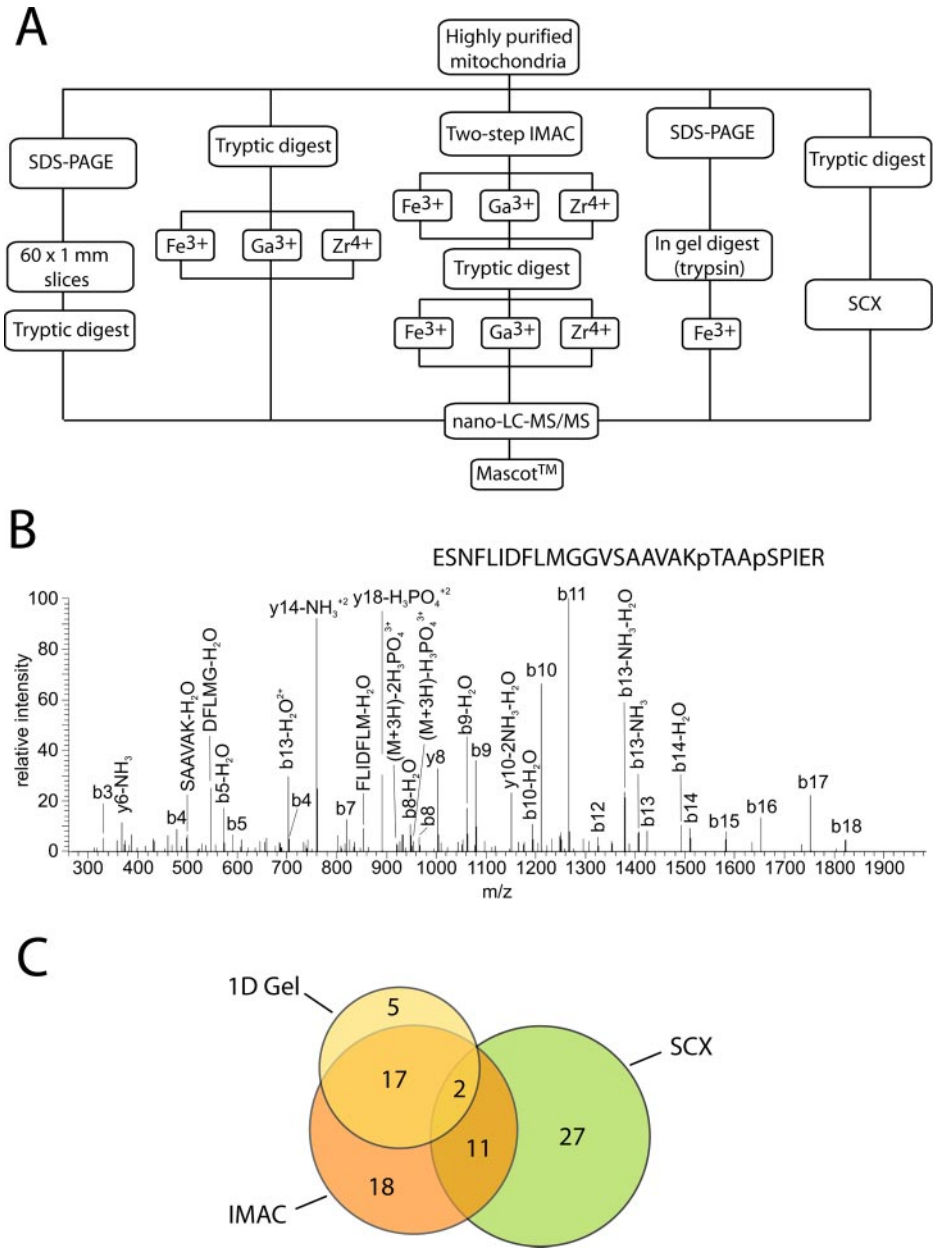

FIG. 1. **Scheme of experimental approaches for identification of mitochondrial phosphoproteins.** *A*, starting with pure yeast mitochondria, several parallel approaches were used as outlined in the figure. The technical details are described under "Experimental Procedures." *B*, fragment ion spectrum of the peptide ESNFLIDFLMGGVSAAVAKpTAApSPIER, derived from Pet9, with two phosphorylation sites. All prominent signals can be assigned to b- and y-ions, internal fragments, and neutral losses. *C*, identification of phosphorylation sites by different methods of phosphopeptide enrichment (as described in *A*). *1D*, one-dimensional.

Only phosphopeptides that were identified at least three times were considered as positive hits. An example for the spectrum of a peptide containing two phosphorylated residues is shown for Pet9 (Aac2), the main ATP/ADP carrier in the mitochondrial inner membrane (Fig. 1*B*).

Using strict criteria for the interpretation and validation of phosphopeptide spectra as outlined under "Experimental Procedures," we identified 80 different phosphorylation sites in 48 proteins (Table I). For 28 proteins, a single phosphorylation site was found. 14 proteins contained two distinct phosphorylation sites that were present in close proximity to each other at the primary structure level in most cases. Six proteins possessed three to six different phosphorylation sites each (Mir1, Atp2, Uip4, Ato2, Ato3, and Jen1). We found 49 phosphoserine residues and 29 phosphothreonine residues (in two cases (Aac1 and Ehd3) the exact position of the phosphorylated residue could not be assigned (Table I)). We did not find phosphorylated tyrosine residues in agreement with previous observations that *S. cerevisiae* does not contain typical tyrosine kinases (10, 61, 62) and only a few cases of phosphotyrosines (39, 63), which are probably generated by dual specificity kinases (61). The absence of phosphotyrosines in this study is not due to technical reasons because we can routinely detect such peptides with our methods in other organisms (64).[2]

[2] R. P. Zahedi, N. Pfanner, C. Meisinger, and A. Sickmann, unpublished data.

TABLE I

Yeast mitochondrial phosphopeptides identified in this study

Phosphorylated peptides from highly purified yeast mitochondria were identified as described under "Experimental Procedures." 80 different phosphorylation sites within 48 different proteins were identified. Asterisks indicate N-terminal acetylation. Spectra are listed in Supplemental Table 1. GPI, glycosylphosphatidylinositol.

ORF	Protein	Gene	Identified phosphopeptide[a]	Position of phosphorylated amino acid[b]
YER178W	Pyruvate dehydrogenase complex E1-α subunit	*PDA1*	YGGHpSMSDPGTTYR	Ser-313
YMR056C	Mitochondrial inner membrane ADP/ATP translocator	*AAC1*	STpSQRQFNGLLDVYK	Ser-155/Thr-156/Ser-157[c]
YBL030C	Major ADP/ATP carrier of the mitochondrial inner membrane	*PET9*	ESNFLIDFLMGGVSAAVAKpTAApSPIER	Thr-39, Ser-42
YJR077C	Phosphate transporter	*MIR1*	LVpSQPQFANGLVGGFSR	Ser-145
			SVpSAAPAIPQYSVSDYMK	Ser-4
			SpTLGCPPTIEIGGGGH	Thr-297
YBL099W	ATP synthase subunit α	*ATP1*	RpSVHEPVQTGLK	Ser-178
			GVpSDEANLNETGR	Ser-57
YJR121W	ATP synthase subunit β	*ATP2*	GIpSELGIYPAVDPLDSK	Ser-373
			EpTGVINLEGESK	Thr-237
			VLDpTGGPISVPVGR	Thr-112
YPL078C	ATP synthase subunit 4	*ATP4*	IDpSVSQLQNVAETTK	Ser-144
YDR298C	ATP synthase subunit 5	*ATP5*	NpSSIDAAFQSLQK	Ser-48
			GpTVTSAEPLDPK	Thr-139
YPL271W	ATP synthase subunit ϵ	*ATP15*	NGpTAASEPTPITK	Thr-52
YDL004W	ATP synthase subunit δ	*ATP16*	RpSYAEAAAASSGLK	Ser-21
YPR020W	ATP synthase subunit g	*ATP20*	QpSLNFALKPTEVLSCLK	Ser-62
			*MLpSRIQNYTSGLVSK	Ser-3
YLR038C	Cytochrome *c* oxidase, subunit VIb	*COX12*	GIFAGDINpSD	Ser-82
YGL187C	Cytochrome *c* oxidase, subunit IV	*COX4*	EGpTVPTDLDQETGLAR	Thr-55
YPR191W	Ubiquinol cytochrome *c* reductase core protein 2	*QCR2*	pSAEDQLYAITFR	Ser-141
YPL262W	Mitochondrial and cytoplasmic fumarase (fumarate hydratase), converts L-malate to fumarate as part of the tricarboxylic acid cycle	*FUM1*	ANEPRIHELLpTK	Thr-428
YDR148C	Component of the mitochondrial α-ketoglutarate dehydrogenase complex	*KGD2*	GLVpTPVVRNAESLSVLDIENEIVR	Thr-340
YOR142W	α subunit of succinyl-CoA synthetase	*LSC1*	SGpTLTYEAVQQTTK	Thr-186
			pSGTLTYEAVQQTTK	Ser-184
YOR136W	Isocitrate dehydrogenase, subunit 2	*IDH2*	pTGDLAGTATTSSFTEAVIK	Thr-349
YKL085W	Malate dehydrogenase	*MDH1*	FIpSEVENTDPTQER	Ser-177
YLR304C	Aconitase 1	*ACO1*	pTIFTVTPGSEQIR	Thr-409
YBL015W	Acetyl-CoA hydrolase	*ACH1*	pSQVVSNNPEMIR	Ser-350
YBR120C	Translational activator of COB mRNA	*CBP6*	MVDEKFpTEESINEQIR	Thr-97
YML120C	NADH-ubiquinone oxidoreductase (AMID homolog)	*NDI1*	FASTRpSpTGVENSGAGPTSFK	Ser-27, Thr-28
YHR008C	Manganese-superoxide dismutase	*SOD2*	LpTNpTKLAGVQGSGWAFIVK	Thr-147, Thr-149
YDR036c	Member of the enoyl-CoA hydratase or isomerase family (fatty acid metabolism)	*EHD3*	LLTKpSPSSLQIALR	Thr-326/Ser-328[c]
YPL186c	Unknown function	*UIP4*	GLDNLpSEGNDNDNTR	Ser-205
			ELpSPNFSQEQTENK	Ser-185
			GNVTFPpSPK	Ser-140
YOR374W	Mitochondrial aldehyde dehydrogenase	*ALD4*	EMpSVDALQNYLQVK	Ser-500
			pTAESTPLSALYVSK	Thr-216
YML128C	Protein of unknown function	*MSC1*	DpTVFDKWpSSDQLTNWLESHK	Thr-237, Ser-243
YPL231W	Fatty-acid synthase α subunit	*FAS2*	QDISSSpTR	Thr-567
YPR184W	Glycogen-debranching enzyme	*GDB1*	DQPLYpTVK	Thr-45
YMR212C	Protein of unknown function	*EFR3*	DNQISpTSDLLSDSQVR	Thr-565
YAR035W	Outer carnitine acetyltransferase, mitochondrial	*YAT1*	LFVKSLLDQDApSDATK	Ser-517

TABLE I—*continued*

ORF	Protein	Gene	Identified phosphopeptide[a]	Position of phosphorylated amino acid[b]
YKL187C	Protein of unknown function	*YKL187C*	KGEIYTYGDGSAIpSADR	Ser-678
			KGEIYTYGDGpSAIpSADR	Ser-675, Ser-678
YKL217W	Pyruvate and lactate/H^+ symporter, member of the major facilitator superfamily (MFS)	*JEN1*	VYpSQDQGVEYEEDEEDKPNLSAASIK	Ser-81
			YINQVEEYEADGLpSISDIVEQK	Ser-584
			*SSpSITDEK	Ser-4
			SSpSITDEKISGEQQQPAGR	Ser-4
			MIDSNVpSK	Ser-606
			SSpSITDEKIpSGEQQQPAGR	Ser-4, Ser-11
YCR010C	Protein may function as an acetate permease, Fun34 motif	*ADY2*	IYpTGGDNNEYIYIGR	Thr-47
YNR002C	Protein possibly involved in ammonium export, Fun34 motif	*ATO2*	ALDpSSEGEFISENNDQSR	Ser-21/Ser-22[c]
			ALDpSSEGEFISENNDQSR	Ser-21, Ser-22
			ALDpSpSEGEFIpSENNDQSR	Ser-21, Ser-22, Ser-28
			HSQEpSICK	Ser-40
			EQpSSGNTAFENPK	Ser-7
			pSDREQSSGNTAFENPK	Ser-2
			ALDpSpSEGEFISENNDQSR	Ser-21, Ser-22
YDR384C	Protein possibly involved in ammonium export, Fun34 motif	*ATO3*	TSSApSpSPQDLEK	Ser-6, Ser-7
			TSpSASSPQDLEK	Ser-4
			TSpSApSSPQDLEK	Ser-4, Ser-6
			TpSSApSSPQDLEK	Ser-3, Ser-6
			TSSApSSPQDLEK	Ser-6
YBR054W	Protein paralog of Mrh1p, has similarity to heat shock protein Hsp30	*YRO2*	AQEEEEDVApTDpSE	Thr-341, Ser-343
			KAQEEEEDVApTDSE	Thr-341
			KAQEEEEDVApTDpSE	Thr-341, Ser-343
YDR033W	Membrane protein related to Hsp30p	*MRH1*	APVApSPRPAATPNLSK	Ser-289
			PAApTPNLSK	Thr-295
YGR086C	Phosphorylation inhibited by long chain bases	*PIL1*	ALLELLDDSPVpTPGETRPAYDGYEASK	Thr-233
YOL109W	Protein that appears to act as a negative regulator of the Pkc1p-Mpk1p cell integrity pathway	*ZEO1*	LEETKEpSLQNK	Ser-25
			NEApTPEAEQVK	Thr-49
			EQAEApSIDNLK	Ser-40
YMR031C	Protein of unknown function	*YMR031c*	NNpSITSATSK	Ser-130
YBL064C	Mitochondrial thiol peroxidase	*PRX1*	INpSDAPNFDADTTVGK	Ser-53
YBR078W	GPI-anchored protein of unknown function	*ECM33*	LQpSNGAIQGDSFVCK	Ser-339
YML072C	Protein involved in calcium-dependent phospholipid binding	*TCB3*	SPSNLNSTSVpTPR	Thr-1350
YJR045C	Mitochondrial heat shock protein 70	*SSC1*	AQFEpTLTAPLVK	Thr-330
YLR259C	Mitochondrial heat shock protein 60	*HSP60*	pTNEAAGDGTTSATVLGR	Thr-102
YNL055C	Porin 1	*POR1*	pSAVLNTTFTQPFFTARG	Ser-109

[a] Phosphorylated amino acid is indicated by p (pS for phosphoserine or pT for phosphothreonine).
[b] Positions were determined from precursor protein sequences.
[c] Manual inspection of spectra revealed ambiguous phosphorylation site.

As shown previously (65), different phosphopeptide enrichment methods reproducibly enrich for distinct segments of the phosphoproteome. In the present study, the overlap between IMAC and SCX, for instance, accounts for only 11 phosphopeptides, whereas 18 phosphopeptides were exclusively identified using IMAC, and 27 were identified only using SCX enrichment (Fig. 1*C*). However, 18 phosphopeptides, which were identified by IMAC but not after SCX enrichment, contain missed cleavage sites and therefore are expected to elute with the bulk of doubly (and triply) charged non-phosphorylated tryptic peptides at pH 2.7.

Functional Classification of Identified Phosphoproteins—We grouped the identified phosphoproteins according to their determined or proposed functions (Fig. 2). The two largest

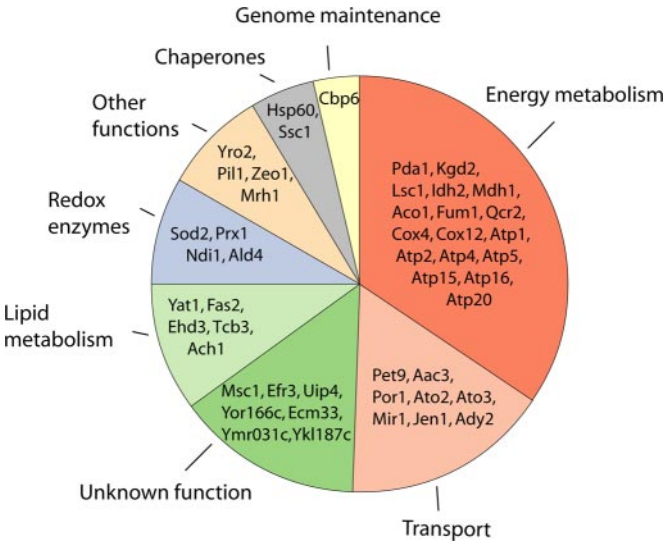

FIG. 2. **Functional classification of mitochondrial phosphoproteins identified in *S. cerevisiae*.** Proteins were classified according to the standard yeast databases, *Saccharomyces* Genome Database (SGD) and Yeast Protein Database (YPD) (77, 78).

groups were formed by proteins involved in mitochondrial energy metabolism and transport processes. Remarkably seven subunits of the F_0F_1-ATPase were phosphorylated, suggesting that this central machinery for cellular ATP production is a frequent target for covalent modification. Among the phosphorylated transport proteins, Ato2, Ato3, and Ady2 belong to the GPR1/FUN34/yaaH family and are potential permeases for acetate and ammonium (66, 67). For Ato2, Ato3, and Yat1 of the lipid metabolism group, a role in mitochondrial retrograde signaling was reported (6). For Pil1 and Zeo1 a role in the Pkc1-MAP kinase pathway has been proposed (68, 69). Further groups of phosphoproteins include proteins involved in redox processes, protein folding, and genome maintenance (Fig. 2). Thus phosphorylation is observed in a broad spectrum of yeast mitochondrial proteins. Furthermore we identified seven phosphoproteins with unknown function, raising the possibility that phosphorylation can affect additional mitochondrial activities.

Phosphorylation of Atp20 Affects Dimerization of the F_0F_1-ATPase—Atp20 has been shown to be required for dimerization of the F_0F_1-ATPase (70–72). Dimerization of the ATP synthase can be directly monitored by blue native electrophoresis of mitochondria lysed with the non-ionic detergent digitonin (Fig. 3*A*, *lanes 1*, *5*, and *9*) (70). The monomeric ATP synthase migrates at ~600 kDa, whereas the dimeric form migrates at ~1200 kDa on a blue native gel, here analyzed with three different antibodies directed against subunits β (Atp2) and γ (Atp3) of the F_1 part and subunit Atp6 of the F_0 part. When mitochondria were isolated from a yeast mutant strain lacking the gene *ATP20*, dimerization was blocked (Fig. 3*A*, *lanes 4*, *8*, and *12*) (70). Re-expression of Atp20 from a plasmid restored dimerization of the ATP synthase (Fig. 3*A*, *lanes 1*, *5*, and *9*). We found that Atp20 was phosphorylated at serine residues 3 and 62 (Table I). We substituted glutamic acid for serine 62 to create a phosphomimetic residue. (We did not modify serine residue 3 because the permanent introduction of a negative charge in the amino terminus of the precursor protein may affect its mitochondrial import (1, 28, 57, 58), raising the possibility of indirect effects.) The mutant protein $Atp20^{S62E}$ was expressed from a plasmid in an *atp20*Δ yeast strain, and mitochondria were isolated. The mitochondria were lysed with digitonin and subjected to blue native electrophoresis. Generation of the dimeric ATP synthase was strongly inhibited in the mutant, whereas the monomeric ATP synthase was efficiently formed (Fig. 3*A*, *lanes 2*, *6*, and *10*). The mutant protein $Atp20^{S62E}$ was imported into isolated mitochondria and transported to a protease-protected location (Fig. 3*B*, *lanes 3* and *4*), excluding the possibility that the mutation prevented transfer of the precursor of Atp20 to mitochondria. The steady-state levels of various control proteins analyzed were comparable to that of wild-type mitochondria, including Atp3 and the preprotein translocase subunits Tim22 and Tim23 of the inner membrane (Fig. 3*C*, *lanes 7–9* compared with *lanes 1–3*). Moreover the TOM complex analyzed by blue native electrophoresis was present as in wild-type mitochondria (Fig. 3*D*, *lanes 1* and *2*). We thus conclude that a phosphomimetic residue at position 62 of Atp20 inhibits formation of the dimeric ATP synthase.

We also substituted alanine for serine 62. The resulting mutant protein $Atp20^{S62A}$ was expressed in *atp20*Δ yeast. Isolated mitochondria were subjected to blue native electrophoresis. The mitochondria efficiently formed the dimeric ATP synthase with a yield that was even moderately increased compared with that of wild-type mitochondria (Fig. 3*A*, *lanes 3*, *7*, and *11*). $Atp20^{S62A}$ was imported into mitochondria to a protease-protected location (Fig. 3*B*, *lanes 5* and *6*) and the steady-state levels of control proteins were comparable to that of wild-type mitochondria (Fig. 3, *C* and *D*). Thus the prevention of phosphorylation of serine 62 of Atp20 favors dimerization of the mitochondrial ATP synthase.

DISCUSSION

We have performed the first systematic profiling of phosphorylation sites of yeast mitochondrial proteins. With a multidimensional approach we identified 80 phosphorylation sites in 48 proteins of highly purified mitochondria. Three previous studies analyzed the phosphoproteome of whole yeast cells. Taken together, Ficarro *et al.* (37) (383 phosphorylation sites in 171 different yeast proteins), Gruhler *et al.* (38) (729 phosphorylation sites in 503 different yeast proteins), and Chi *et al.* (39) (1252 phosphorylation sites in 629 different yeast proteins) found only 10 mitochondrial phosphoproteins that were identified here. Thus, the subfractionation of yeast to purify mitochondria significantly increased the sensitivity of detection of phosphoproteins.

An important point is the inclusion of phosphatase inhibi-

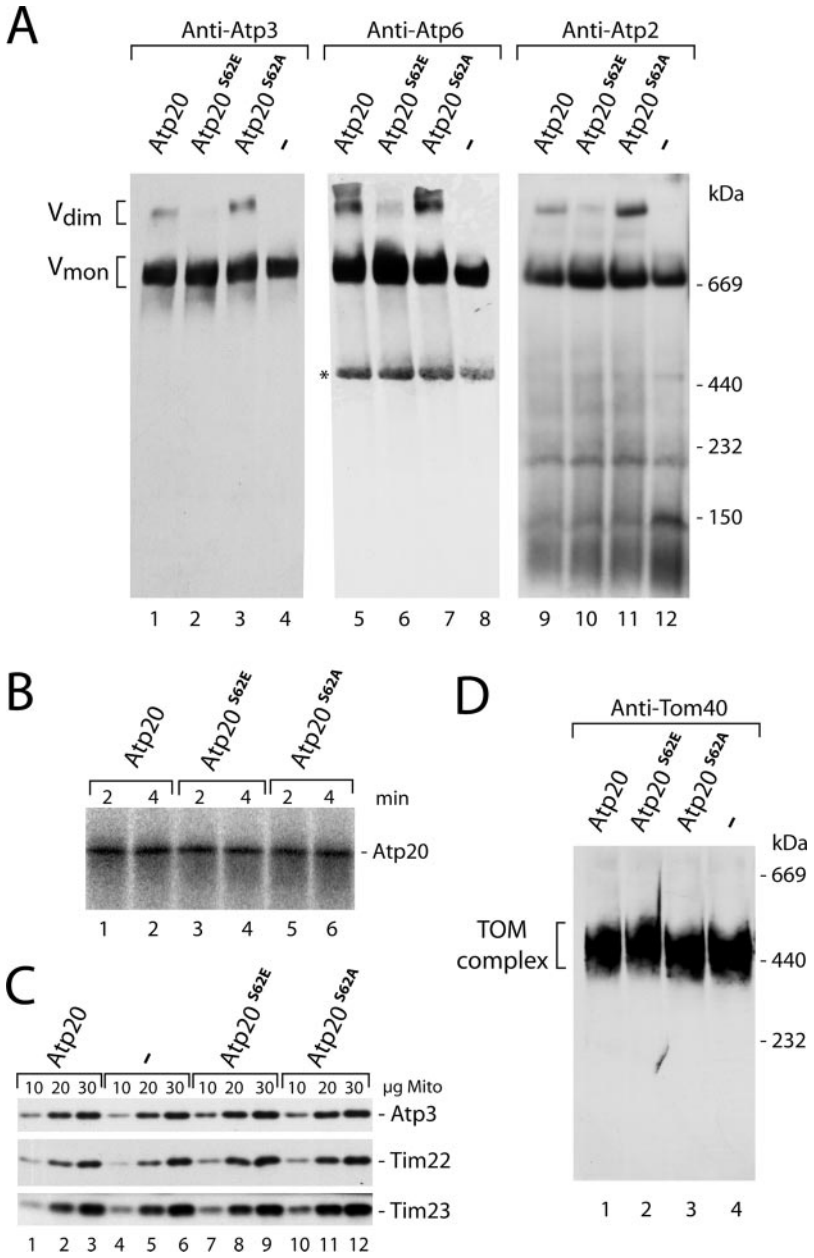

FIG. 3. **Phosphorylation of Atp20 interferes with dimerization of the mitochondrial ATP synthase.** *A*, analysis of monomeric (V_{mon}) and dimeric ATP synthase (V_{dim}) by blue native electrophoresis. Mitochondria isolated from yeast *atp20*Δ strains expressing either wild-type Atp20, Atp20^{S62E}, Atp20^{S62A}, or the empty vector (−) were lysed in digitonin buffer and subjected to blue native electrophoresis. Immunodecoration was performed using the indicated antisera and the ECL detection system. By comparing the levels of dimeric ATP synthase in the presence of Atp20^{S62A} (non-phosphorylated), Atp20^{S62E} (phosphomimetic), and wild-type Atp20, the functional degree of phosphorylation was assessed to be ~40% under wild-type conditions. *Asterisk*, unspecific protein band (79). *B*, import of mutant forms of Atp20 into mitochondria. Radiolabeled Atp20 with wild-type sequence or S62E or S62A mutations were imported into isolated mitochondria for the indicated time periods as described under "Experimental Procedures." After treatment with proteinase K, mitochondrial proteins were separated by SDS-PAGE, and imported proteins were visualized by digital autoradiography. *C*, steady-state levels of proteins in mitochondria with mutant Atp20. Mitochondrial proteins were separated by SDS-PAGE and blotted onto PVDF membrane. Immunodecoration was performed with the indicated antisera and the ECL detection system. *D*, the TOM complex is not altered by mutation of Atp20. Mitochondria were lysed in digitonin buffer and separated by blue native electrophoresis followed by Western blot analysis with Tom40 antisera. *Mito*, mitochondria.

tors during the subfractionation of yeast cells because in the absence of added inhibitors we were only able to identify 10 phosphoproteins in isolated mitochondria. It is conceivable that additional mitochondrial phosphoproteins were not detected in our study. Possible reasons are that not all phosphatases were inactivated during the cellular subfractionation, that only a minor fraction of a protein may be phosphorylated under the growth conditions applied, that the tryptic digest failed to generate peptides that were reliably detected by MS, or that the presence of further modifications within a phosphopeptide, introduced *in vitro* as well as *in vivo*, might interfere with the analysis (*e.g.* we identified several amino-terminal phosphopeptides that were acetylated).

The identified phosphoproteins cover a broad range of mitochondrial functions from bioenergetics, transport, and lipid metabolism to redox regulation, protein folding, and genome maintenance (Fig. 2), suggesting that many mitochondrial functions are regulated by reversible protein phosphorylation. The identification of seven phosphoproteins with so far unknown function supports this view. Phosphorylation is likely to play a critical role in the communication of mitochondria with the nucleus because Ato2, Ato3, and Yat1, three proteins with a role in retrograde signaling of mitochondria (6), were found to be phosphorylated. Interestingly Pil1 and Zeo1, which are involved in the Pkc1-MAP kinase pathway (68, 69), are also phosphorylated. The Pkc1-MAP kinase pathway regulates cell wall integrity and cell proliferation (68, 69, 73), raising the question of how this pathway should be related to mito-

chondria. Very recently, it was shown that upon exposure to farnesol Pkc1 could localize to yeast mitochondria where it is involved in managing the generation of reactive oxygen species most likely by phosphorylation of mitochondrial proteins (74).

Seven subunits of the F_0F_1-ATPase were found to be phosphorylated (Table I). A detailed analysis of subunit g (Atp20) by genetic manipulation and a native gel assay revealed that phosphorylation of serine 62 plays a critical role in the dimerization of the ATP synthase. Generation of a phosphomimetic residue at position 62 (S62E) inhibited dimerization of the ATP synthase, whereas a block of phosphorylation by substitution of alanine for serine even enhanced the level of dimerization. It has been reported that a conserved G*XXX*G motif in the transmembrane domain of Atp20 is involved in oligomerization of the ATP synthase (71, 72). Similarly such a motif was also observed in the transmembrane domain of subunit e (Atp21/Tim11), a second dimerization factor of the ATP synthase (75). Because an intact G*XXX*G motif is not essential for the interaction of Atp20 with Atp21, further regions of these subunits seem to be involved in the interaction (72). The phosphorylation site described here is located in the matrix domain of Atp20, *i.e.* outside the transmembrane domain, and may thus mark an additional region involved in dimerization. As oligomerization of the ATP synthase is important for a full bioenergetic activity of mitochondria (76), phosphorylation of Atp20 is apparently involved in regulating the bioenergetic state of mitochondria.

Because yeast is a major model organism for studying mitochondrial functions and for the analysis of human mitochondrial diseases, the phosphoproteome reported here will provide a rich source for characterizing the role of reversible phosphorylation in regulation of mitochondrial activities under physiological and pathological conditions and in communication of mitochondria with the rest of the cell.

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