The activity of yeast pyruvate dehydrogenase complex is regulated by reversible phosphorylation. Recently, we identified two enzymes that are involved in the phosphorylation (Pkp1p) and dephosphorylation (Ppp1p) of Pda1p, the α-subunit of the pyruvate dehydrogenase complex. Here we provide evidence that two additional mitochondrial proteins, Pkp2p (Ygl059wp) and Ppp2p (Ycr079wp), are engaged in the regulation of this complex by affecting the phosphorylation state of Pda1p. Our data indicate complementary activities of the kinases and a redundant function for the phosphatases. Both proteins are associated with the complex. We propose a model for the role of the regulatory enzymes and the phosphorylation state of Pda1p in the assembly process of the pyruvate dehydrogenase complex.

The mitochondrial pyruvate dehydrogenase complex (PDC) with a size of ~8 MDa consists of multiple copies of three catalytic subunits (E1–E3) (1–3). This multienzyme complex connects glycolysis and the tricarboxylic acid cycle by catalyzing the oxidative decarboxylation of pyruvate to acetyl-CoA. As is the case for many other rate-limiting, flux-generating metabolic reactions, PDC activity is tightly regulated (4–7). This is achieved by reversible phosphorylation by a concerted activity of pyruvate dehydrogenase kinases and phosphatases (8, 9). In mammals, four kinase isomers (pyruvate dehydrogenase kinases 1–4) and two phosphatase isomers (pyruvate dehydrogenase phosphatases 1 and 2) have been described. They act in a tissue and phosphorylation site-dependent manner and differ in their efficiency to (de-)phosphorylate the α-subunit of E1 (4, 10, 11). Phosphorylation of either of three serine residues (Ser264, Ser271, Ser203 (16, 17)) or a combination thereof leads to differential inactivation of the enzyme to a certain extent, whereas dephosphorylation restores activity (4, 12). Phosphorylation of site 1 (Ser264) correlates with the strongest inactivation (13). The number of phosphorylation sites varies between organisms; whereas three sites are found in many different species of the animalia, plants contain only one site, and a variable number is present in Fungi and Protista (10). Although all putative phosphorylation sites are conserved in the yeast Saccharomyces cerevisiae (Sc), only phosphorylation at site 1 (S(Ser)264, corresponds to Ser264 in mammals) has been reported (14, 15). Mitochondrial phospho-proteomic studies so far did not identify a Pda1p-derived peptide bearing a phosphorylation at the other sites (16–19). In contrast to the mammalian enzyme, in Sc the PDC kinase and phosphatase are not tightly bound to the complex and escaped their identification for a long time. Recently, our group used a reverse genetic approach to identify two enzymes engaged in phosphorylation (Pkp1p) or dephosphorylation (Ppp1p) of Pda1p (20). Both enzymes are associated with PDC in a not yet defined manner. Interestingly, cells lacking the kinase Pkp1p accumulate active PDC subcomplexes, suggesting an additional role of the kinase and/or the phosphorylation in PDC assembly (20). However, the yeast genome encodes an additional putative kinase (Ygl059wp) in the mitochondrial proteome (21), which shares 42% homology to Pkp1p. Because its functional role is currently unknown, we included Ygl059wp in our study on PDC phosphorylation.

The previously identified PDC-phosphatase Ppp1 (20) belongs to the evolutionary conserved protein phosphatase family type 2C and, thus, has been originally named Ptc5p as one of seven members (Ptc1p–Ptc7p) (22). Ptc1p–Ptc4p are localized to the nucleus and/or the cytoplasm (23, 24), whereas Ptc5p (Ppp1p), Ptc6p (Ycr079wp), and Ptc7p (Yhr076wp) have been assigned to the mitochondrial compartment (20, 23, 25), suggesting a role in dephosphorylation of mitochondrial proteins. Therefore, we included Ycr079wp and Yhr076wp in our survey for enzymes engaged in dephosphorylation of PDC.

We report here on the analysis of the respective null mutants and demonstrate that two of the investigated proteins, Ygl059wp and Ycr079wp, are involved in the phosphorylation and dephosphorylation of PDC in yeast, respectively. In analogy to the previously identified enzymes, we propose to name these new enzymes as PDC kinase II (Pkp2p) and PDC phosphatase II (Ppp2p).

Pda1p, the α-subunit of PDC, was identified as the target of phosphorylation by means of two-dimensional isoelectric focusing (IEF)/SDS-PAGE, phosphospecific in gel fluorescence staining, and subsequent mass spectrometry. In addition, we present data on the molecular organization and association of the regulatory enzymes with PDC. We discuss that the PDC phosphatases show partial functional redundancy, whereas the PDC kinases exhibit complementary activity.
Regulation of Yeast PDC

EXPERIMENTAL PROCEDURES

Strains and Media—Sc wild type strain BY4741 (accession number Y00000) and deletion strains \(\Delta pda1\) (accession number Y06174), \(\Delta yil042c\) (accession number Y01435), \(\Delta ygl059w\) (accession number Y04426), \(\Delta ypr090c\) (accession number Y01866), \(\Delta ycr079w\) (accession number Y05798), and \(\Delta ylr076w\) (accession number Y01904) were obtained from Euroscarf. Strain expressing tandem affinity purification (TAP)-tagged Pkp1 (YIL042c-TAP, accession number YSC1178-7501003) was purchased from Open Biosystems. Yeast complete media contained 1% yeast extract, 2% peptone, and 2% glucose or 3% ethanol as the sole carbon source. Yeast minimal media were prepared as described (26).

Epitope Tagging—Fusion of proteins with either c-Myc- or HA tag was achieved by homologous recombination of the corresponding integration cassettes (27) in the chromosomal loci of strains BY4741, \(\Delta ygl059w\), \(\Delta ycr079w\), or YIL042c-TAP. Integration cassettes for c-Myc or HA tagging were PCR-amplified from vector pGA2254 (containing nine repeats of c-Myc epitope) or vector pWZV108 or pUC19-3HA-URA (containing three repeats of HA epitope), respectively (kind gifts of W. Zachariae, MPI-CBG Dresden, Germany).

Growth Analysis by Nephelometry—Growth of yeast cells was monitored in 96-well plates. 200 \(\mu\)l of the respective medium per well were inoculated with \(10^3\) cells of an overnight culture in YPD. Plates were incubated at 30 °C with constant shaking, and light scattering was measured in 20-min intervals in a NEPHLOstar (BMG Labtech).

Isolation and Purification of Mitochondria—Yeast cells were grown to early stationary phase in media containing ethanol as the carbon source. Isolation and purification of mitochondria by two successive sucrose gradient centrifugations and treatment with 1 \(M\) NaCl were performed as described (28, 29). Phosphatase inhibitor cocktails I + II (Sigma) were included in a 1:100 dilution throughout the preparation.

Proteinase K Treatment of Mitochondria and Mitoplasts—

For mitoplast formation 50 \(\mu\)g of mitochondria were incubated in ice-cold hypotonic buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.1 M mannitol) and incubated for 30 min on ice. Mitoplasts were stabilized by the addition of 1 volume buffer containing 0.65 M mannitol. Proteinase K was added to a final concentration of 0.5 \(\mu\)g/\(\mu\)l to either mitochondria or mitoplasts in the presence or absence of 1% Triton X-100 (Roche Applied Science). Samples were incubated for 15 min on ice, and the reaction was stopped by the addition of 1 \(M\) 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (Applichem). The pellet obtained by centrifugation (20,000 \(\times\) g for 10 min at 4 °C) was subjected to SDS-PAGE. Samples lysed with Triton X-100 were precipitated with trichloroacetic acid (10% final concentration).

Two-dimensional IEF/SDS-PAGE—Two-dimensional PAGE was essentially performed as described (30). 500 \(\mu\)g of highly purified mitochondria were sedimented (12,000 \(\times\) g for 10 min at 4 °C) and lysed in 100 \(\mu\)l of thiourea buffer containing 4% CHAPS for 10 min on ice. The lysate was centrifuged (13,000 \(\times\) g for 10 min at 4 °C) and diluted with 400 \(\mu\)l of thiourea buffer (containing 4% CHAPS and 0.002% bromophenol blue). Samples were applied by in-gel rehydration of Immobiline DryStrips (24 cm, pH 3–11 NL, GE Healthcare) overnight at room temperature. IEF was performed in the Etten IPGphor II system (GE Healthcare) in 3 steps (1 h at constant at 200 V, 2 h gradient up to 1000 V, and a further 6 h constant at 8000 V) at 20 °C with 50 \(\mu\)A per strip. Strips were equilibrated for 15 min in SDS buffer containing 10 mg/ml dithiothreitol and finally in SDS buffer with 25 mg/ml iodoacetamide (30). The second dimension was carried out in the Etten DALTSix system (GE Healthcare) using 12% SDS-gels. All chemicals were from GE Healthcare except for iodoacetamide (Sigma), Tris, and glycine (Applichem).

Phosphoprotein and Total Protein Staining—Fluorescent staining of phosphoproteins was performed with ProQ®-Diadmond Stain (Invitrogen) using an optimized protocol (31). Deep Purple total protein staining (GE Healthcare) was carried out according to the manufacturer’s protocol. Imaging of the stained gels was performed with the Typhoon 9410 image scanner (GE Healthcare).

Mass Spectrometry—Protein bands were excised, washed, in-gel reduced, S-alkylated, and in-gel-digested with trypsin (Promega) as described previously (32). MALDI-MS measurements were performed using an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in reflectron mode and \(\alpha\)-cyano-4-hydroxy-cinnamic acid as matrix. Spectra acquisition and processing were done as described previously (33). Protein identification was performed using MASCOT (Matrix Science) and SwissProt (216,380 sequences). Search criteria were as follows: taxonomy, S. cerevisiae; peptide and MS/MS tolerance, 50 ppm and 0.5 Da, respectively; maximum one missed cleavage site; variable modifications, carbamidomethylation, methionine oxidation, phosphorylation of Ser, Thr, and Tyr. For nanoliquid chromatograph-MS/MS experiments, extracted peptides were separated on a Tempo nanomultidimensional liquid chromatograph system (Applied Biosystems) equipped with a RP-C18 trap column and a PepMap C18 analytical column (75 \(\mu\)m inner diameter \(\times\) 150 mm) directly coupled to the micro-ion spray II nano-source of a 4000 Q TRAP system (Applied Biosystems). Peptides were eluted with a 30 min linear gradient of 5–95% acetonitrile in 0.1% formic acid at 250 nl/min. All MS was conducted in positive mode using 2700 eV source voltage, sheath gas and curtain gas of 10 (arbitrary units), and an interface heater temperature of 150 °C. A multiple reaction monitoring-initiated detection and sequencing scan (MIDAS) was developed for all potential phosphorylation sites (Ser(P), Thr(P), and Tyr(P)) of Pda1p using either neutral loss of \(H_3PO_4(–98)\) or the Tyr(P) immonium ion \((m/z\) 216). A scan of 156 multiple reaction monitoring transitions triggered an enhanced resolution scan of the three most intense ions followed by enhanced product ion scans if the ion intensity was greater than a threshold value. Precursor ions were subjected to collision-induced dissociation using rolling collision energy, and the \(m/z\) of the ion was determined from the enhanced resolution scan. Enhanced product ion scans were searched using MASCOT and SwissProt. Peptide and MS/MS tolerance were set to 0.5 Da (other search criteria were as above). Phosphopeptides identified by MASCOT were manually sequenced, and spectra were analyzed by ProteinPilot (Applied Biosystems).
The Protein Phosphatases Ppp2p and Yhr07w6p and the Protein Kinase Pkp2p Are Localized in the Mitochondrial Compartment—We have previously identified two enzymes that phosphorylate (Pkp1p) and dephosphorylate (Ppp1p) Pda1p, thereby regulating the activity of PDC. However, the yeast genome encodes additional candidate proteins of PDC regulation; Ppk2p is 42% homologous to Pkp1p, whereas Ppp1p shares 49 and 37% homology with Ppp2p and Yhr07w6p, respectively. We first addressed the question of whether these proteins are targeted to mitochondria, which is an essential prerequisite for enzymes directly affecting the phosphorylation state of mitochondrial PDC. To this end, the respective reading frames were fused at the carboxyl terminus either with the c-Myc- or the HA-tag coding sequence (see “Experimental Procedures”). To assess the subcellular localization of the proteins, crude mitochondria were isolated and subjected to two consecutive sucrose gradient purification steps (Fig. 1A). To remove proteins that are associated with the cytoplasmic face of the mitochondrial outer membrane, aliquots of the gradient-purified mitochondria were treated with 1 M NaCl. All fusion proteins tested showed an identical fractionation profile to those of the mitochondrial control proteins aconitase (Aco1p, mitochondrial matrix), cytochrome c oxidase subunit II (Cox2p, inner mitochondrial membrane), and the intermembrane space protein cytochrome b2 (Cyb2p). In contrast, the highly abundant cytoplasmic phosphoglycerate kinase (Pgg1p), which is firmly attached to the outer surface of the mitochondrial outer membrane, was no longer detectable in the salt-washed fraction. This suggests the complete removal of cytoplasmic contaminations. No (Pkp2p, Ppp2p) or only a very faint (Yhr07w6p) signal was detected in the cytoplasmic fraction, indicating that these proteins are almost exclusively localized within the mitochondrial compartment as was also shown for the previously identified PDC kinase Pkp1p and PDC phosphatase Ppp1p (Fig. 1A).

Interestingly, in a recent report Ppp2p (therein denoted as Aup1p) was found to be localized in the mitochondrial intermembrane space and to be involved in the process of mitophagy (38). This localization would preclude the direct interaction and dephosphorylation of the target protein Pda1p in the

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**FIGURE 1. Mitochondrial localization of the phosphatases Ppp1p, Ppp2p, and Yhr07w6p and of the kinases Pkp1p and Pkp2p.** A, mitochondria of strains expressing tagged versions of the respective proteins were isolated, and samples were taken from the cytoplasmic (cytoplasm) and mitochondrial fraction before (crude) and after (gradient) purification by two successive sucrose gradients. Purified mitochondria were treated with 1 M NaCl under osmotic stabilizing conditions (NaCl/P), and the supernatant was precipitated with trichloroacetic acid (NaCl; S). 50 μg of each fraction were subjected to SDS-PAGE. B, mitochondria and mitochondria of cells expressing Ppp2p-HA were treated with 0.5 μg/μl of proteinase K in the presence or absence of 1% Triton X-100 and analyzed by SDS-PAGE. Immunological detection was performed with HA or c-Myc antibodies, and detected with horseradish peroxidase-conjugated secondary antibodies and the ECL-Plus kit (GE Healthcare). TAP antibodies (Open Biosystems) are directed against the calmodulin binding domain.

**TAP Method—** The TAP method was performed with 1 mg of mitochondrial proteins of a strain co-expressing Pkp1p-TAP and Pkp2p-HA as described previously (20).

**Co-immunoprecipitation—** 40 μg of HA or c-Myc (Roche Applied Science) antibodies, respectively, were covalently coupled on NHS HP Spin Trap columns (GE Healthcare) as described in the manufacturer’s protocol. 1 mg of mitochondria of a strain co-expressing Pkp1p-c-Myc and Pkp2p-HA were lysed in buffer containing 4% digitonin (10 mM NaCl, 5 mM 6-aminoacaproic acid, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1× proteinase inhibitor mixture (EDTA-free, Roche Applied Science), and phosphatase-inhibitor mixture I + II (Sigma) 1:100). After extensive washing steps, elution was done with 2% SDS.

**SDS-PAGE and Western Blot Analysis—** Preparation of 12% SDS gels and protein electrophoresis was carried out according to Laemmli (34). Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore), probed with primary antibodies, and detected with horseradish peroxidase-conjugated secondary antibodies and the ECL-Plus kit (GE Healthcare). Primary antibodies were directed against c-Myc (Roche Applied Science), HA (Roche Applied Science), Cox2p (cytochrome c oxidase subunit II; Invitrogen), Aco1p (aconitase; a kind gift of R. Lill, Marburg, Germany), Pgp1p (phosphoglycerate kinase; Invitrogen), Cyb2p (cytochrome b2), and Ccp1p (cytochrome c peroxidase; kind gifts of W. Neupert, Munich, Germany). TAP antibodies (Open Biosystems) are directed against the calmodulin binding domain.

**Blue Native (BN)-PAGE—** The method of BN-PAGE (35, 36) was adapted for the separation of salt-labile PDC as previously described (20).

**PDC Activity Assay Based on NADH Production**—The activity of PDC in isolated mitochondria was determined as described by James et al. (37). 40 μg of mitochondrial protein

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### Table: Mitochondrial and Mitoplast Localization of PDC Kinases and Phosphatases

| Protein   | Mitochondria | Mitoplast |
|-----------|--------------|-----------|
| Aco1p     | -            | +         |
| Cyb2p     | -            | +         |
| Ccp1p     | +            | -         |

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### Figure 1A

- **A**
  - A scheme showing the fractionation of mitochondrial proteins into cytoplasmic and mitochondrial fractions before and after gradient purification.
  - The proteins are categorized into mitochondrial or cytoplasmic association.

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### Figure 1B

- **B**
  - A gel representation showing the immunoblot analysis of proteins from mitochondria and mitoplasts.
  - Proteins are detected with antibodies specific to HA or c-Myc, revealing their association with either fraction.

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**Regulation of Yeast PDC**

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Regulation of Yeast PDC

Mitochondrial matrix. To clarify this issue we reinvestigated the submitochondrial localization of Ppp2p. Mitoplasts were generated from gradient-purified mitochondria of cells expressing Ppp2p-HA and subjected to proteinase K treatment (Fig. 1B). In line with the results shown above, Ppp2p is resistant to proteinase K digestion of intact mitochondria. Complete digestion of the protein upon solubilization of the mitochondrial membranes by Triton X-100 indicates that the protein per se is sensitive to proteinase K (Fig. 1B). After rupturing the outer mitochondrial membrane by hypo-osmotic treatment, Ppp2p is still detectable in presence of proteinase K and thus, behaves like the matrix protein aconitase. In contrast, the marker proteins of the intermembrane space Cyb2p and Ccp1p are degraded, documenting the accessibility of this compartment for the proteinase K. We conclude that Ppp2p is a protein of the mitochondrial matrix. This is supported by the in silico prediction of a 33-amino acid-residue-long cleavable mitochondrial presequence (39).

FIGURE 2. PDC activity assay of wild type and deletion strains of mitochondrial protein kinases and phosphatases. Mitochondria of wild type, the kinase deletions strains Δpck1 and Δpck2, and the phosphatase deletion strains Δppp1, Δppp2, and Δyhr076w were isolated. The Δpda1-strain was included as negative control. PDC activity was measured by following the NADH formation (A600) over time (average of three replicates). Relative activities were determined by calculating the slopes of the different measurements in the linear range within the first 90 s. Activity of wild type (7.9 units/mg of mitochondrial protein) was set to 100% (S.D. is given in bars).

Activity of PDC Is Regulated by Ppp2p and Pkp2p but Not by Yhr076w—We next studied the influence of deletions of the respective mitochondrial kinase and phosphatase genes on PDC activity in cells grown on ethanol. Wenzel et al. (40) showed an unexpected high activity of PDC under aerobic growth on ethanol. Mitochondria of the null mutant strains were isolated and tested for PDC activity by monitoring the production of NADH in the presence of pyruvate as a substrate (Fig. 2). No enzymatic activity was detectable in strains lacking the PDC subunit Pda1p, documenting the specificity of the assay. As previously described (20), deletion of PPP1 decreased the activity by 56%, whereas the absence of PDC kinase Pkp1p does not significantly change the activity compared with wild type under the growth conditions used (Fig. 2). Surprisingly, PDC activity in PPP2 deletion strains dropped dramatically to 6%. In contrast, in the absence of Pkp2p, PDC possessed a 45% higher activity compared with wild type. These results strongly argue that both enzymes are involved in the regulation of PDC. No alterations in enzyme activity were observed for the yhr076w null mutant, suggesting that this phosphatase is not engaged in PDC regulation.

Deletion of Either of the PDC Phosphatases Affects Growth—We compared the growth in the presence of different carbon sources of the wild type and the pda1 deletion strain with that of strains lacking either of the regulatory PDC enzymes (Fig. 3). As expected, cells devoid of an active PDC due to the absence of Pda1p possessed a reduced growth rate and a lower final cell density on glucose and ethanol (41). Similar phenotypes were observed for strains lacking either of the phosphatases on media with ethanol due to the diminished PDC activity in these strains described above. The cell division rate is most notably reduced in the case of Δppp2. This finding is in line with the more severe reduction of PDC activity upon deletion of PPP2. However, this residual activity in both Δppp2 and Δppp1 is sufficient to allow for the production of wild type-like final cell densities on glucose. Interestingly, only in Δppp2 the adaptation phase is elongated on glucose. This may hint at an additional regulatory function of Ppp2p (see “Discussion”).

Pda1p Is the Target of Phosphorylation—To identify the target protein(s) of Pkp2p and Ppp2p, mitochondrial proteins of

Growth analysis of wild type and deletion strains. Growth kinetics (average of three replicates) of the indicated strains on full media containing either glucose (A) or ethanol (B) as the sole carbon source was monitored in 96-well plates by measuring the light scattering in a Nephelometer. 200 μl of medium per well were inoculated with the respective saturated culture in YPD to yield a constant starting cell density of 10^4 cells/well. Plates were shaken at 30 °C, and measurement was performed in 20-min intervals. wt, wild type.
wild type and deletion strains were separated by two-dimen-
sional IEF/SDS-PAGE (Fig. 4A) and subsequently stained with
the phosphospecific fluorescent dye ProQ-Diamond (Fig. 4B,
lower panel), with a fluorescent total protein stain (Fig. 4B,
upper panel), and finally with Coomassie Brilliant Blue (data
not shown) to allow for spot picking. Comparison of the gels
revealed one spot (Fig. 4B, spot 1) that significantly differed in
the staining intensity; although stained in wild type, Δppp1, and
Δppp2 strains, no signal can be detected in strains lacking
Pkp1p, Pkp2p, and Pda1p. The respective protein of spot 1 was
identified by MALDI TOF/TOF as Pda1p. In line with this, the
respective spot was missing in the mitochondrial proteome of
Δpda1. To identify the phosphorylation site(s) of Pda1p, a
MIDAS method for 156 multiple reaction monitoring transi-
tions covering potential Ser(P)-, Thr(P)-, and Tyr(P)-contain-
ning peptides was applied. Thus, a monophosphorylated pepti-
de (retention time 5.78 min) was detected, and fragment ion anal-
ysis revealed the sequence \(^{309}\text{YGGHpSMSDPGTTYR}^{322}\) (pS is
phosphorylated Ser; Fig. 4C). Phosphorylation at position 313
of the peptide was confirmed by the b6 and y10 ions showing
neutral loss of \(\text{H}_3\text{PO}_4\), whereas the y8 and y9 ions did not. This
excludes phosphorylation at position 315 and unambiguously
assigns the phosphorylation to serine 313, which is in line with
the findings of previous publications (14, 17, 19). In addition to
this most abundant phospho-signal, two other spots were visi-
table (Fig. 4B, spots 2 and 3). However, these proteins were not
detectable by Coomassie staining and, hence, could not be iden-
tified by MALDI-MS. Because these spots have an almost identi-
cal molecular weight (M) as spot 1 and behave similarly with
respect to the phospho-staining intensity in the various genetic
backgrounds, we assume that they represent Pda1p variants of
different pI values. This interpretation is supported by compar-
ing our two-dimensional data with those of Ohlmeier et al. (42).
These authors could identify Pda1p variants at virtually the
same pI and \(M\) region as calculated for spots 1–3 (Fig. 4B). No
other protein except for Pda1p was identified in the spot corre-
sponding to spot 1.\(^3\)

As expected, no Pda1p phospho-signals were detected in the
absence of the PDC kinase Pkp1p (Fig. 4B). Concomitantly, in
the total protein staining the signal corresponding to spot 1 was
absent, which is likely due to the shifted pI value as a result of
the missing phosphate group. In contrast, deletion of Ppp1p
resulted in an enhanced phospho-signal and protein concentra-
tion of spot 1. Interestingly, deletion of Pkp2p and Ppp2p led to
alterations of the phospho and total protein pattern that are
identical to those described for strains lacking Pkp1p and
Ppp1p, respectively. This is in line with our data on the influ-
ence on PDC activity and clearly suggests that Pkp2p inacti-
vates PDC by phosphorylation of Pda1p, whereas dephospho-

\(^3\) S. Ohlmeier, personal communication.
rlation by Ppp2p is associated with activation of the complex. Taken together, our findings provide strong evidence that yeast PDC is regulated by more than one kinase and phosphatase. Each of the two PDC kinases alone is able to virtually completely dephosphorylate Pda1p. The extent of Pda1p phosphorylation in strains lacking one of the phosphatases is difficult to quantify since the dephosphorylated (basic) Pda1p form is most likely superposed by the very abundant Cor1p (core protein of the bc1 complex of the respiratory chain; Fig. 4B, spot 4 (42)). Cor1p is slightly visible in the phospho-staining presumably due to the tendency of the phospho dye to unspecifically bind to high abundant proteins (43).

Partially Overlapping Native Separation Profiles of Pkp2p and Ppp2p Suggest Similar but Not Identical Molecular Organizations—Both Pkp1p and Ppp1p are associated with PDC (20). To study this issue for the newly identified enzymes, the distribution of their HA-tagged variants of both proteins were analyzed by two-dimensional BN/SDS-PAGE of mitochondrial lysates and compared with that of Pda1p (Fig. 5A). Due to the size of the fully assembled PDC (8 MDa), which exceeds the pore size of the 3% gel, the complex is retained in the stacking gel (20, 44). Assemblages in the molecular mass range of 100–500 kDa possibly reflect the already described PDC subcomplexes (Fig. 5A, region I) as was previously reported for the deletion of Pkp1p (20), could not be observed. Presumably, Pkp2p and Ppp2p do not encompass functions in the biogenesis of PDC.

Pkp1p and Pkp2p Interact—The very similar BN-PAGE separation profiles can hint at a physical interaction of Pkp1p and Pkp2p. To test this, we analyzed the mitochondrial lysate of a strain that concomitantly expressed a TAP-tagged Pkp1p and a HA-tagged Pkp2p. The lysates were subjected to the two consecutive steps of the tandem affinity purification (Fig. 6A). As expected, Pkp1p-TAP was precipitated by both the first IgG affinity matrix (Fig. 6A) and the second calmodulin matrix. The molecular mass shift results from the release of the protein A domain (16 kDa) upon cleavage by the tobacco etch virus protease. Interestingly, Pkp2p-HA was coprecipitated on both matrices, whereas the mitochondrial proteins Cox2p and Aco1p were not. This result clearly indicates that Pkp1p and Pkp2p interact.

The interaction between the two kinases was independently confirmed by a co-immunoprecipitation approach. Mitochondrial lysates of cells co-expressing Pkp1p-c-Myc and Pkp2p-HA were incubated with either immobilized HA or c-Myc antibodies (Fig. 6B). The eluate fraction of the HA antibodies column contained both Pkp2p-HA and Pkp1p-c-Myc. In the reciprocal experiment Pkp1p-c-Myc as well as Pkp2p-HA was detected in the precipitate of the c-Myc antibody column. Specificity of the results is documented by the failure to detect Cox2p and Aco1p by the respective antibodies. Overall our experiments show that Pkp1p and Pkp2p physically interact in vivo, possibly by formation of heteromers.

Regulation of Yeast PDC

FIGURE 5. Molecular organization of PDC, Pkp2p, and Ppp2p. High molecular weight complexes were separated by BN-PAGE in a 3–13% gradient gel. Subunit composition of these complexes was assessed by a second dimension under denaturing conditions (12% SDS-gel). Proteins were analyzed by Western blot and immunologically detected with HA antibodies. Signals of Pda1p-HA indicate the position of fully (8 MDa, stacking gel) and partially (~50–1400 kDa, region I and II) assembled PDC. Signals of the membrane protein Cox2p document solubilization of mitochondrial membranes and serve as internal size standard by marking the positions of the respiratory supercomplexes of complexes III and IV (S1, [III]2[IV]1 at ~500 kDa; S2, [III]2[IV]2 at ~1100 kDa). A, molecular organization of Pkp2p and Ppp2p was analyzed using 200 μg of mitochondrial proteins from strains expressing Pkp2p-HA and Ppp2p-HA. B, molecular organization of PDC in strains lacking either Pkp2p or Ppp2p was analyzed in mitochondrial lysates of wild type (wt), Δppp2, and Δpkp2 strains expressing Pda1p-HA and detected with HA-antibodies.
Regulation of Yeast PDC

yeast cells to be sensitive to rapamycin (45). To test whether this phenotype results directly from the loss of Ppp2p function or is due to secondary effects, we compared the growth in the absence and presence of rapamycin of the Δpplp2 strain with that of wild type and of Δpda1 and Δppplp1 strains (Fig. 7). As expected, the Δpda1 strain showed a slightly reduced growth on YPD without rapamycin, whereas growth of the phosphatase deletion strains was indistinguishable from that of wild type. In the presence of rapamycin, growth of both Δppplp2 and Δpda1 was markedly inhibited, contrary to wild type cells which were resistant up to 3 ng/ml rapamycin. Δppplp1 showed only a slightly reduced growth, likely due to the higher residual PDC activity (see above).

DISCUSSION

The PDC couples two major energy-generating pathways, glycolysis and the tricarboxylic acid cycle. In mammals, PDC is tightly regulated via phosphorylation and dephosphorylation by a coordinated activity of four tissue-specific PDC kinases and two PDC phosphatases, which are firmly attached to the complex (for review, see Ref. 46). These regulatory enzymes themselves are regulated by a variety of conditions, including the intramitochondrial concentrations of cofactors and substrates or products of PDC reaction (1, 3, 7, 11, 47–49), indicating a complex multilayer regulatory network. Failure of the human PDC (50) or of one of its regulatory enzymes (51, 52) results in serious disorders. In yeast, however, the conversion of pyruvate to acetyl-CoA can be bypassed by two additional mitochondrial and cytoplasmic pathways (53, 54). Hence, loss of PDC function can be tolerated and leads (dependent on the genetic background) only to a slight phenotype (54). As in mammals, PDC in S. cerevisiae is regulated by reversible phosphorylation of its Pda1p subunit on Ser313 (14). Contrary to the mammalian system, the yeast regulatory enzymes of PDC are only loosely and/or transiently bound and, therefore, escaped from co-purification and identification for a long time. Only recently we were able to identify Pkp1p and Ppp1p as regulatory kinase and phosphatase of PDC, respectively (20). Pkp1p is one of ten atypical protein kinase in yeast and belongs to the pyruvate dehydrogenase kinase family (55, 56). Analyses of the yeast genome revealed a second member of this family, Pkp2p, which shares 45% homology with Pkp1p. Here, we provide experimental evidence that Pkp2p is a mitochondrial protein partially associated with the PDC and also involved in its regulation. In the absence of Pkp2p, phosphorylation of Ser313 of Pda1p (Fig. 4, spot I) is no longer detectable by the phospho-specific staining.

Although this observation suggests that Pkp2p is a kinase of Pda1p, some observations hint at a different function of the protein. Despite its high degree of homology, Pkp2p differs from Pkp1p in some important aspects; mitochondrial protein kinases are characterized by five highly conserved regions that presumably define the putative catalytic domain (for review, see Ref 57). Some of the invariant residues in these subdomains, including His148 in subdomain I, which is directly involved in the catalytic phosphate transfer reaction (58), are conserved in Pkp1p but not in Pkp2p. Furthermore, the glycine-rich loops of subdomains III and V that form part of the nucleotide binding pocket (59) are perfectly conserved in PDC-kinases but not in Pkp2p. However, experimental evidence in favor of a kinase activity of Pkp2p comes from an in vitro study with heterologously expressed glutathione S-transferase fusion proteins of yeast kinases (56). It is questionable whether these results also hold true for the protein in vivo.

Our data show that both Pkp1p and Pkp2p seem to possess complementary functions in Pda1p phosphorylation. Because only a single phosphorylation site is known in yeast Pda1p, a concerted and cumulative mechanism of kinase activities on different target sites as in the mammalian system (see introduction) obviously does not exist in yeast. However, it has been shown for the mammalian PDC, that all four kinase isoforms are able to phosphorylate site 1 (which corresponds to Ser313 in yeast), albeit with different efficiencies. Pyruvate dehydrogenase kinase 2 possesses the highest activity followed by the isoforms 4, 1, and 3 (10). If Pkp2p would represent a kinase whose

FIGURE 6. Physical interaction of Pkp1p and Pkp2p. Interaction of both kinases were analyzed by TAP (A) and co-immunoprecipitation (B). For the TAP method, 1 mg of mitochondrial protein of cells co-expressing Pkp1p-TAP and Pkp2p-HA were lysed (20 μg, lysate) and incubated with the first affinity matrix (IgG beads). After extensive washing (1/10 of IgG bead vol, IgG), cleavage with tobacco etch virus protease was performed, and the supernatant was subjected to the second affinity step on calmodulin beads. Calmodulin beads were pelleted and washed, and bound proteins were eluted with 2% SDS (cal). Unbound proteins were precipitated (unbound). Samples were separated by SDS-PAGE, and proteins were detected by HA and TAP antibodies. 

FIGURE 7. Effect of rapamycin on growth of wild type (wt) and PDC phosphatase deletion strains (Δpppl, Δppplp2). The indicated strains were grown overnight in YPD, and a dilution series of 104–101 cells was dropped on YPD plates without or with 3 ng/ml rapamycin. Growth was documented after 2 days (YPD) or 4 days (YPD + rapamycin) at 30 °C.
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function overlaps with Pkp1p, one would expect Pda1p phosphorylation in the PKP2 deletion strain due to the presence of the second kinase. However, phosphorylation of Ser313 is completely impeded upon deletion of either of the proteins. Perhaps their concomitant activity is needed to compensate for the high dephosphorylation rate by the PDC phosphatases. However, we consider this possibility unlikely, as the energy demand of this type of regulation would be enormous. Instead, we favor the idea that both proteins interact to stimulate Pda1p phosphorylation. Our experimental data clearly document that both kinases interact in vivo, possibly forming heteromeric complexes. Physical interaction of mammalian members of the PDC kinase is known albeit only to form functional homodimers. Hetero-oligomer formation of Pkp1p and Pkp2p might be required to mediate association with the PDC (sub)complexes. Contrary to the situation in mammals, where pyruvate dehydrogenase kinases are activated via conformational changes upon binding to the lipoyl domains of PDC, yeast kinases do not contain such a binding domain. Perhaps heteromeric Pkp1p/Pkp2p complexes provide the basis for the attachment to the PDC. Alternatively, interaction of both kinases may result in mutual phosphorylation and activation.

Interestingly, the consequences of deletion of either Pkp1p or Pkp2p on PDC activity are not identical; we observe 145% “over”-activated in the absence of Pkp2p, whereas PDC activity is not markedly altered in the case of Pkp1p deletion under the conditions tested. This result suggests that their mode of function differs. Indeed, studies of the molecular organization of PDC revealed that lack of Pkp1p, but not of Pkp2p, results in the accumulation of enzymatic active subcomplexes (20). Eventually this observation can account for the diminished overall PDC activity. Recently, we proposed that Pkp1p may also play a role in the process of PDC assembly either via phosphorylation or by the presence of the protein itself (20). In view of our present data that deletion of Pkp2p results in a completely dephosphorylated PDC without affecting complex integrity, we assume that binding of the kinase to the subcomplexes rather than the phosphorylation of Pda1p is important for the PDC assembly.

The two kinases differ in respect to their molecular organization. As previously described, Pkp1p is preferentially associated with the PDC subcomplexes of about 400 kDa, and only a small portion of the protein is bound to the fully assembled complex (20). In contrast, Pkp2p is detected predominantly as part of complexes in the molecular mass area of 500–2000 kDa with additional signals merging those of Pkp1p and Pda1p. The strong abundance of Pkp2p may indicate that these complexes are phosphorylated, and hence, inactive. We propose a model of coordinated assembly and activity regulation in yeast. In the mammalian PDC it is suggested that the kinases move in a “hand-over-hand” mechanism by binding/detaching from the flexible lipoyl tail that allows for an efficient phosphorylation of all E1 subunits of the complex by a few molecules (12). In yeast, however, the kinases are presumably not bound via the lipoyl moiety, and thus, a similar moving mechanism is unlikely to occur. We propose that in yeast a pool of inactive pre-complexes exists that can be activated on demand for assembly to functional PDC to meet the required metabolic criteria. Fine-tuning of its activity is achieved by a balanced assembly and disassembly of active (not phosphorylated) and inactive (phosphorylated) PDC subcomplexes. The identified complexes in the molecular mass range of 500–2000 kDa could, thus, represent the regulation and assembly scaffold of PDC.

In our previous paper (20) we proposed a second PDC phosphatase that accounts for the residual activity of PDC in strains lacking Ppp2p. Here we provide experimental evidence for Ppp2p as a second mitochondrial PDC phosphatase. It belongs to the class of type 2C phosphatases, and its enzymatic activity has been documented in vitro on artificial substrates (45). Deletion of PPP2 results in a dramatic decrease of PDC activity (to 6% residual activity) and is accompanied by hyperphosphorylation of Pda1p. Under this condition the residual ability of the cells to convert pyruvate to acetyl-CoA is likely due to the activity of Ppp1p, reflecting its lower dephosphorylation rate. As already discussed above, the molecular organization of the phosphatases is similar and likely reflects association with the fully and partially assembled PDC.

Unexpectedly, our phenotypical analysis revealed that growth on glucose and ethanol was more strongly affected in Δppp2 than in a strain devoid of an active PDC (Δpda1). This may hint at an additional regulatory function of Ppp2p. The result is interesting in light of the fact that different functions have been implicated for this protein in previous studies. It was reported that Ppp2p is involved in the process of mitophagy (38) and confers a rapamycin-resistant function (45), and its deletion results in a caffeine-sensitive phenotype (60). We assume that these pleiotropic phenotypes are likely due to the critical role of PDC in the metabolism by supplying acetyl-CoA. Alterations in PDC activity may eventually lead to metabolic constraints, and this may subsequently result in pleiotropic reactions of the cells by initiation of a variety of stress responses. We show that cells lacking Ppp2p but also cells with a non-functional PDC due to deletion of PDA1 possess similar sensitivity toward rapamycin, indicating that impaired energy metabolism rather than the deletion of Ppp2p is the reason. Our study revealed that Ppp2p is almost exclusively localized in the mitochondrial matrix and associated with PDC. Although we cannot exclude that a minor portion of Ppp2p is localized outside of the mitochondrial matrix, participation in a mitochondrial matrix to cytoplasmic signaling during mitophagy as proposed by Tal et al. (38) seems unlikely. A role of Ppp2p in mitophagy may be due to the inactivation of PDC in cells lacking Ppp2p and/or due to the impairment of the mitochondrial energy metabolism. Mitophagy may be a means of the cell to cope with the metabolic requirements in the absence of functional PDC by generating amino acids, fueling the tricarboxylic acid cycle and maintaining the ATP production (61). However, as outlined above, our experiments indicate an alternative function for Ppp2p that remains to be elucidated.

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