Isoenzymes of Pyruvate Dehydrogenase Phosphatase
DNA-DERIVED AMINO ACID SEQUENCES, EXPRESSION, AND REGULATION

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Pyruvate dehydrogenase phosphatase (PDP) is one of the few mammalian phosphatases residing within the mitochondrial matrix space. It is responsible for dephosphorylation and reactivation of the pyruvate dehydrogenase complex (PDC) and, by this means, is intimately involved in the regulation of utilization of carbohydrate fuels in mammals. PDP is a dimeric enzyme consisting of catalytic and regulatory subunits. The catalytic subunit of PDP is a Mg\(^{2+}\)-dependent enzyme homologous to the cytosolic phosphatases of the 2C family. In the present study, we isolated two cDNAs encoding for mitochondrial phosphatases. The first cDNA is highly homologous to the previously identified cDNA encoding for the catalytic subunit of PDP (PDP1). The second cDNA encodes a previously unknown catalytic subunit of PDP (PDP2). The new phosphatase, expressed as the recombinant protein in *Escherichia coli*, shows strict substrate specificity toward PDC and does not use phosphorylated branched chain \(\alpha\)-ketoadid dehydrogenase as substrate. Like PDP1, PDP2 is a Mg\(^{2+}\)-dependent enzyme, but its sensitivity to Mg\(^{2+}\) ions is almost 10-fold lower than that of PDP1. In contrast to PDP1, PDP2 is not regulated by Ca\(^{2+}\) ions. Instead, it is sensitive to the biological polyamine spermine, which, in turn, has no effect on the enzymatic activity of PDP1.

Western blot analysis of PDP extracted from mitochondria isolated from liver and skeletal muscle revealed that PDP1 is predominantly expressed in mitochondria from skeletal muscle, whereas PDP2 is much more abundant in the liver rather than muscle mitochondria. Both isoenzymes are expressed in mitochondria from 3T3-L1 adipocytes, but the level of expression of PDP2 is considerably higher. These observations are consistent with previous findings on the enzymatic parameters of PDP in adipose tissue. Thus, our results provide the first evidence that there are at least two isoenzymes of PDP in mammals that are different with respect to tissue distribution and kinetic parameters and, therefore, are likely to be different functionally.

The reaction catalyzed by the mammalian pyruvate dehydrogenase complex (PDC)\(^1\) links glycolysis with several biochemical pathways. In tissues with large energy demands like brain, muscle, and heart, it supplies the carbon units derived from carbohydrate fuels mainly for complete oxidation by the Krebs cycle. In lipogenic tissues such as adipose, mammary gland, and liver, the metabolic fate of acetyl-CoA derived from carbohydrates is quite different, because it can be used for the biosynthesis of fatty acids and cholesterol (for reviews, see Refs. 1 and 2). Therefore, the reaction catalyzed by PDC should be highly regulated, and this regulation must be sophisticated enough to accommodate the different metabolic requirements of a variety of tissues.

Indeed, several molecular mechanisms have been implicated in the regulation of mammalian PDC. In the short term, the enzyme activity may be regulated by negative feedback (3, 4). The products of the PDC reaction, NADH and acetyl-CoA, inhibit the overall reaction by reversing the partial reactions catalyzed by the dihydrolipoamide dehydrogenase and transacylase components of the complex (4). This mechanism may be important for the rapid adjustment of flux through PDC, particularly in the liver (3). A perhaps even more important mechanism of PDC regulation is based on reversible phosphorylation (5). Mammalian mitochondria contain two enzymes dedicated to the regulation of PDC: pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP) (6). The kinase is an integral part of the multi-enzyme complex (7). It phosphorylates three serine residues of the decarboxylase component (pyruvate decarboxylase) (8). Phosphorylation completely halts the enzymatic activity of PDC (9, 10). The phosphoenzyme can be reactivated only via dephosphorylation catalyzed by phosphatase (11). In contrast to PDK, PDP is loosely associated with PDC but becomes complex-bound upon stimulation (12). Both PDK and PDP can integrate a considerable number of different regulatory stimuli (reviewed in Ref. 13). The kinase activity is likely to reflect the relative intramitochondrial concentrations of the substrates (pyruvate, NAD\(^{+}\), and CoA) and products (NADH and acetyl-CoA) of the main dehydrogenase reaction. An excess of substrates results in the inhibition of kinase activity with concomitant activation of PDC by the phosphatase. An excess of products activates the kinase and results in the net phosphorylation and inactivation of PDC. The enzymatic activity of PDP depends on intramito-

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\(^1\) The abbreviations used are: PDC, pyruvate dehydrogenase complex; PDP, pyruvate dehydrogenase phosphatase; PDP1, previously identified cDNA encoding for the catalytic subunit of PDP; PDP2, previously unidentified cDNA encoding for the catalytic subunit of PDP; PDK, pyruvate dehydrogenase kinase; P2C, phosphoprotein phosphatase 2C; kb, kilobase(s); bp, base pair(s); Mops, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.
Isoenzymes of Pyruvate Dehydrogenase Phosphatase

Polymerase Chain Reaction—Common PCR primers for amplification of PDP-related genes were designed according to the sequences of polypeptides -A20-TDGLEW35- and -M442-YRDDIT448- of bovine PDP (GCN ACN GA/CT) GGN CTG TGG GA and GT(A/G) AT/AG) and bovine PDP, respectively. These primers were used to amplify rat liver QUICK-Clone™ cDNA. The PCR reaction mixture contained 50 pmol of each gene-specific primer and 1 ng of double-stranded cDNA as well as deoxyribonucleic triphosphates, buffer, and 5.0 units of native Pfu DNA polymerase, which were added according to the manufacturer’s instructions. 35 cycles of PCR were set up using 1 min at 94 °C for denaturation, 1 min at 50 °C for annealing, and 1 min at 72 °C for extension. The resulting PCR product of approximately 300 bp was subcloned in pUC18 and sequenced. The cDNA for bovine PDP was amplified by reverse transcriptase-PCR using reverse-transcribed total RNA from bovine heart as the template. The upstream (GCT TCT ACA CCG CAG AAG TTT) and downstream (CTG TCT CTT GTG ATA TGG) PCR primers corresponded to the bases 378–398 and 1757–1778 of published cDNA. Reverse transcriptase-PCR reactions were set up according to the manufacturer’s instructions. The first strand was primed with random hexamers. The cycling parameters were as follows: 1 min at 94 °C for denaturation, 1 min at 60 °C for annealing, and 3 min at 72 °C for extension with a 10-min final extension at 72 °C. The resulting DNA (1.4 kb) was subcloned in pUC18 and sequenced.

Rapid Amplification of the 5'-End of PDP2 cDNA (5'-RACE)—Commercially available 5'-RACE-Ready™ templates prepared from rat liver cDNA were used in order to obtain the 5'-end of PDP2 cDNA. The first round of amplification was set up according to the manufacturer’s instructions with gene-specific primer (TAG GTC AAG GAG TTC CTG CCA ATA) corresponding to bases 663–686 of PDP2 cDNA. For the second round of amplification, gene-specific primer (CCA CGT CAG GAT GGG CAG CAA) corresponding to positions 558–596 was used. Several PCR products ranging in size from 300 to approximately 700 bp were separated on TAE-agarose gel, purified, and subcloned in pUC18 for sequencing. The PCR products obtained from three independent amplifications were analyzed.

Construction of the Bacterial Expression Vectors for Rat PDP1 and PDP2 cDNAs—NcoI and XhoI restriction sites flanking the coding region of PDP1 cDNA corresponding to the mature polypeptide were constructed by PCR using the cDNA of rat PDP1 (cloned in the present study) as template and native Pfu DNA polymerase as primer (AAA CCA TGG TCT CTA CGG CTC AGA AAT TGG ATC) containing the NcoI restriction site (both restriction sites are underlined) corresponded to bases 340–363 of rat PDP1 cDNA. The antisense primer (TTT CTC GAG TTC TCT GTG ATG ATA TGG) carrying the XhoI restriction site corresponded to bases 1720–1740. The resulting cDNA was digested with NcoI and XhoI restriction enzymes and subcloned between the NcoI and XhoI sites of the pET-28a expression vector in order to produce the carboxyl-terminal fusion with His tag encoded by the vector (plasmid PDP1). The cDNA for rat PDP2 was amplified using primers AAA TCG TCA TCA CAT CAA CCA AGG AAG AAG AT (bases 225–247) and TTT CCT GAG ACC CTC CTA CCA ATG TTG ATC (bases 1597–1617). The sense primer contained the BspHI restriction site, and the antisense primer contained the XhoI restriction site (both restriction sites are underlined). Amplification reactions were performed using rat liver QUICK-Clone™ cDNA as the template with native Pfu DNA polymerase. The resulting cDNA of approximately 1.4 kb was digested with BspHI (New England BioLabs) and XhoI restriction enzymes and subcloned between the NcoI and XhoI sites of pET-28a (plasmid PDP2). The resulting plasmid directs the synthesis of PDP2 with a carboxyl-terminal His tag. The fidelity of the correct pPDI and pPDP2 vectors was established by nucleotide sequencing.

Nucleotide Sequencing—Sequencing of double-stranded plasmid DNA was carried out by the Biochemistry Biotechnology Facility (Indiana University) using a T7 DyeDeoxy terminator cycle sequencing kit with AmpliTaq DNA polymerase, F9 (Perkin-Elmer) following the manufacturer’s instructions. Both strands were sequenced.
Expression and Purification of Recombinant Proteins—To establish expressing cell lines, competent BL21(DE3) cells were co-transformed with one of the pPDP vectors and pGroESL containing the inducible genes for the molecular chaperons GroEL and GroES. Resulting transformants were selected on TY agar plates containing kanamycin (45 μg/ml) and chloramphenicol (35 μg/ml). The cells were grown in 37 °C in an incubator with shaking at 300 rpm. The cell density and temperature were monitored by measuring the optical density at 600 nm. The OD reached 0.5–0.6, the flasks were still transferred to a shaker at room temperature and induced with 0.4 mM IPTG. After the OD of the culture at 600 nm reached 0.5–0.6, the flasks were still transferred to a shaker at room temperature and induced with 0.4 mM IPTG. The recombinant phosphatases, 10 μl of the respective glycerol stock were used to prepare glycerol stocks. In order to express the recombinant phosphatases, 10 μl of the respective glycerol stock was inoculated into 1 liter of M9ZB medium containing kanamycin (45 μg/ml) and chloramphenicol (35 μg/ml). The cells were grown at 30 °C in a shaker at 300 rpm. After induction for 20–24 h at room temperature, the cells were harvested by centrifugation at 5000 rpm (JA-10 rotor) for 20 min at 4 °C. The pellets were suspended in 10 volumes of TN buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM NaCl) supplemented with 10 mM β-mercaptoethanol, 0.5% Triton X-100, and a mixture of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 20 μg/ml leupeptin, 20 μg/ml pepstatin A, and 1% (v/v) aprotinin). Cells were disrupted by sonication (five times for 20 s with 1-min intervals for cooling on ice). The extracts were clarified by centrifugation at 50,000 × g for 30 min at 4 °C. To purify recombinant proteins, the extracts were desalted on PD-10 columns (Amersham Pharmacia Biotech) equilibrated with 25 mM Mops, pH 7.3, 0.1 mM EDTA, 0.1% β-mercaptoethanol, and 10% glycerol (MEMG buffer) and immediately used without any further purification. For raising antiserum, both PDP1 and PDP2 were further purified on a 5-ml bed volume of TALON resin equilibrated with 3 volumes of TN. After loading of the extract, the column was sequentially washed with 10 volumes of TN and 5 volumes of TN containing 15 mM imidazole. Bound phosphatase was eluted with TN plus 100 mM imidazole. Fractions containing phosphatase were pooled, supplemented with dithiothreitol, the mixture of protease inhibitors given above, and glycerol (50% final concentration). Resulting preparations were stored in small aliquots at −80 °C.

Preparation of Phosphorylated Substrate—32P-Labeled substrate was prepared by incubating human recombinant PDC (2–5 mg) in MEMG buffer supplemented with 2.5 mM MgCl2, 0.1 mM [γ-32P]ATP (specific activity ~1,000 cpm/pmol) and recombinant PDK3 (10–25 μg) in a final volume of 1.0 ml at 37 °C for 30 min. After the end of the incubation, the reaction was terminated with polyethylene glycol-8000 (12% final concentration) for 1 h on ice. The precipitate was collected by centrifugation at 12,000 rpm for 10 min at 4 °C, dissolved in 1.0 ml of MEMG, and passed through a PD-10 column equilibrated with MEMG to remove the radioactive ATP. The resulting complex contained 4–9 nmol of phosphorl group/mg of protein.

PDP Assay—The PDP activity assay was performed essentially as described by Yan et al. (22). Briefly, the extract containing PDP was diluted in MEMG buffer containing 1 mg/ml bovine serum albumin, and aliquots containing approximately 1.0 μg of total protein were preincubated at 30 °C for 2 min with the indicated concentrations of MgCl2 and effectors. The reaction was initiated by adding 32P-labeled PDC (final concentration, 1.0 mg/ml) in a final volume of 40 μl. The reaction was allowed to proceed for 90 s and then terminated with 200 μl of 20% trichloroacetic acid. Precipitated protein was removed by centrifugation at 12,000 rpm for 2 min. Aliquots of the supernatant were counted in order to determine the PDP activity. All experiments were conducted in duplicate. Under the conditions described, the time course of the reaction was linear for several minutes of incubation, and the rate of the reaction was proportional to the amount of PDP added. The results of kinetic experiments were fitted and analyzed using GraFit software (Erithacus Software).

Antigens for Immunoblotting—Antisera against recombinant PDP1 and PDP2 were produced in New Zealand White rabbits following a standard immunization protocol (28). Antisera obtained were specific for each of these isoenzymes and showed little if any cross-reactivity under the loading and detection conditions used in the present study for immunoblotting. Western blot analysis was conducted essentially as described previously (29). Briefly, the mitochondria purified from rat liver were solubilized in 50 mM Tris-HCl, pH 6.8, 1.0 mM EDTA, 2% (w/v) SDS at 100 °C for 5 min. Unsolubilized proteins were removed by centrifugation at 12,000 × g for 10 min at room temperature. Mitochondrial proteins (~10 μg of total mitochondrial protein/lane) were separated on SDS-polyacrylamide gel electrophoresis according to Laemmli (30). Separated proteins were transferred to a nitrocellulose membrane. Blots were blocked in 50 mM Tris-HCl, pH 7.5, containing 0.5 mM NaCl and 0.05% (w/v) Tween 20 (TBST) plus 5.0% (w/v) bovine serum albumin for 2–4 h at room temperature and then incubated with anti-PDP1 or anti-PDP2 antisera diluted 1:1000 in TBST/bovine serum albumin at 4 °C for 10–12 h. Antibody-antigen complexes were visualized after incubation with 125I-labeled goat anti-rabbit IgG in TBST/bovine serum albumin followed by autoradiography. Under the described conditions, there was a linear relationship between the relative densities of the bands corresponding to PDP1 or PDP2 and the amount of total mitochondrial protein loaded per lane.

RESULTS AND DISCUSSION

Molecular Cloning of Rat PDP1 and PDP2 cDNAs—Results from several laboratories have indicated that in mammals, and possibly in other higher eukaryotes, there may be more than one gene encoding for the phosphatase regulating the activity of PDC (23–25). These studies prompted us to search for genes that are homologous to the bovine PDP characterized recently (21). Thus, we employed a PCR-based approach using common primers designed according to the sequences of the two polypeptides (A34TDGLWE351 and M442YRDDIT446). These sequences were chosen based on the recently solved three-dimensional structure of phosphoprotein phosphatase 2C (PP2C) (36). The bovine PDP is a metal-dependent enzyme showing some sequence similarity to PP2C, as pointed out by Lawson and colleagues (21). This suggested that PDP may have a folding pattern reminiscent of other metal-dependent phosphatases (36). The two polypeptides chosen for the primer design in this study are likely to be a part of the bimetallic metal-binding center, residues Asp347 and Asp445 of bovine PDP, and, therefore, should be conserved among PDP-related proteins (see the model below). The use of these primers has an additional advantage, because, in contrast to the cytosolic phosphatases, PDP has a 56-amino-acid long insertion in this region. This, therefore, allows the identification of PDP-related cDNAs according to their size (approximately 300 bp). As discussed under “Experimental Procedures,” amplification of rat liver cDNA indeed yielded a product 300 bp long. Sequencing of this cDNA revealed that it encodes for the protein, showing about 60% similarity to the previously reported sequence of bovine PDP. This finding indicated that there may be another isoenzyme of PDP in mammals or, alternatively, that the rat enzyme is quite distinctly related to the bovine one.

To explore these possibilities, we undertook a series of cloning experiments designed to obtain the cDNA encoding the entire polypeptide of a new PDP-like protein (PDP2) as well as to check whether rodents have a gene analogous to the bovine PDP (PDP1). In order to clone the full-length cDNA encoding rat PDP1, a heart cDNA library was screened with bovine cDNA obtained by PCR. As reported previously by Lawson et al. (21), for unknown reasons the cDNA for PDP1 is underrepresented in commercially available libraries. This appeared to be the case for the rat cDNA as well. Screening of approximately 2–4 × 106 plaque-forming units of the cDNA library resulted in just one positive clone. Fortunately, the resulting clone was 2295 bp long and contained the entire coding region of rat PDP1 (Fig. 1A). The putative translation initiation signal at position 12,000 for 10 min at 4 °C was counted in order to determine the PDP activity. All experiments were conducted in duplicate. Under the conditions described, the time course of the reaction was linear for several minutes of incubation, and the rate of the reaction was proportional to the amount of PDP added. The results of kinetic experiments were fitted and analyzed using GraFit software (Erithacus Software).

Antisera and Immunoblotting—Antisera against recombinant PDP1 and PDP2 were produced in New Zealand White rabbits following a standard immunization protocol (28). Antisera obtained were specific for each of these isoenzymes and showed little if any cross-reactivity under the loading and detection conditions used in the present study for immunoblotting. Western blot analysis was carried out essentially as described previously (29). Briefly, the mitochondria purified from rat skeletal muscle, or 3T3-L1 adipocytes were solubilized in 50 mM Tris-HCl, pH 6.8, 1.0 mM EDTA, 2% (w/v) SDS at 100 °C for 5 min. Mitochondrial proteins (~10 μg of total mitochondrial protein/lane) were separated on SDS-polyacrylamide gel electrophoresis according to Laemmli (30). Separated proteins were transferred to a nitrocellulose membrane. Blots were blocked in 50 mM Tris-HCl, pH 7.5, containing 0.5 mM NaCl and 0.05% (w/v) Tween 20 (TBST) plus 5.0% (w/v) bovine serum albumin for 2–4 h at room temperature and then incubated with anti-PDP1 or anti-PDP2 antisera diluted 1:1000 in TBST/bovine serum albumin at 4 °C for 10–12 h. Antibody-antigen complexes were visualized after incubation with 125I-labeled goat anti-rabbit IgG in TBST/bovine serum albumin followed by autoradiography. Under the described conditions, there was a linear relationship between the relative densities of the bands corresponding to PDP1 or PDP2 and the amount of total mitochondrial protein loaded per lane.

Other Techniques—3T3-L1 cells purchased from ATCC were maintained and differentiated as described by Kedishvili et al. (31). Mitochondria from skeleton muscle, liver, and 3T3-L1 adipocytes were isolated according to Refs. 32–34, respectively. Protein concentration was determined according to Lowry et al. (35).
Fig. 1. Nucleotide and deduced amino acid sequences of rat PDP1 and PDP2. The protein sequence of PDP1 (A) is numbered according to the sequence of the mature polypeptide (ASTPQKF-) as determined by Lawson et al. (21) (underlined). The mitochondrial targeting sequence of PDP2 (B) is proposed to correspond to amino acid residues 267 to 211. The amino acid sequence that is likely to correspond to the mature polypeptide of PDP2 (STEEEDFH-) is underlined. The isoenzymes of Pyruvate Dehydrogenase Phosphatase (17683)
respective signal in the bovine sequence (21). The 3'-noncoding region of the cloned cDNA is 619 bp long and lacks an apparent polyadenylation signal.

The cDNA encoding rat PDP2 was cloned by screening a rat liver library with the respective 300-bp-long PCR product. Three positive clones were identified after analysis of 0.5–1 × 10^6 plaques. The resulting cDNAs ranged in size from 0.7 to 1.2 kb and lacked the 5'-noncoding as well as part of the 5'-coding regions. Further attempts to clone a cDNA encoding at least the entire coding region failed to generate fragments longer than those that had already been obtained. Thus, we used 5'-RACE in order to clone the rest of the PDP2 cDNA. As discussed under “Experimental Procedures,” this approach yielded a cDNA of approximately 700 bp that contained the missing 5'-end. The entire cDNA of rat PDP2 was reconstructed by aligning the nucleotide sequence of the longest DNA fragment (1.2 kb) obtained from the library with the DNA fragment (0.7 kb) obtained via 5'-RACE. As shown in Fig. 1B, the resulting 1746-bp-long cDNA has a potential translation initiation signal at base 27 and a translation termination signal at base 1617, allowing us to deduce the entire protein sequence of PDP2. The 3'-noncoding region of the resulting cDNA is rather short, spanning only 129 bp.

The results of the cloning experiments described above provide strong evidence that there are at least two genes coding for isoenzymes of PDP. It should be pointed out that similar genes are likely to be present in other mammalian genomes and especially in the human genome. A search of the GenBank™ expressed sequence tag data base revealed several expressed sequence tags corresponding to human PDP1 and at least one expressed sequence tag (accession number M85599) corresponding to human PDP2 or a highly related protein.

**Primary Structures of PDP1 and PDP2**—The cDNAs of PDP1 and PDP2 predict polypeptides of 538 and 530 amino acids long with calculated molecular masses of 61,207 and 59,654 Da, respectively. Like most mitochondrial proteins, PDP1 contains the mitochondrial targeting sequence at its amino terminus, which is cleaved after translocation into the mitochondrial matrix space (37). PDP1 has a relatively long leader peptide of 71 amino acids. Its cleavage should generate the mature protein with a predicted molecular mass of 52,618 Da (the amino acid sequence of the mature polypeptide of PDP1 as determined by Lawson et al. (21) for bovine enzyme is underlined in Fig. 1A). Unfortunately, the isoenzyme PDP2, in contrast to PDP1, has never been purified from native sources, and thus, its amino-terminal sequence is currently unknown. Some predictions, however, can be made based on analysis of its primary structure. As shown in Fig. 1B, the sequence of the first 65–70 amino acids of PDP2 is almost completely devoid of negatively charged amino acids and enriched in positively charged residues, which are spread along the entire 70-amino acid-long peptide. These features are consistent with the idea that this region comprises the mitochondrial targeting sequence of PDP2 and should direct the polypeptide into the mitochondrial matrix space (37). Since Western blot analysis suggests that the molecular weight of PDP2 is close to the molecular weight of PDP1 (see below), it is likely that the length of the PDP2 leader peptide is approximately 65–69 amino acids (the putative amino-terminal sequence of the mature polypeptide of PDP2 is underlined in Fig. 1B).

Alignment of the deduced protein sequences of PDP1 and PDP2 revealed approximately 55% identity within the sequences of the mature polypeptides. In several regions that are presumably functionally important, the identity reaches up to 90% (residues 72–94, 163–180, 196–228, 318–351, 404–417, and 432–448 of PDP1). On the other hand, the similarity to the homologous phosphatase 2C is only about 20% (Fig. 2). Like PDP1, PDP2 has a sequence reminiscent, although imperfect, of the EF-hand motif (residues 173–184 for both isoenzymes) (21), which has been implicated in the regulation of PDP activity by Ca^{2+} ions. It also has several insertions that are characteristic of PDP versus the PP2C family (Fig. 2). Thus, comparison of the deduced polypeptide sequences of PDP1 and PDP2 clearly indicates that these enzymes are structurally related.

**Hypothetical Three-dimensional Structure of PDP**—Considerable progress was made recently in the elucidation of the structure and function of metal-dependent phosphatases when Das et al. (36) reported the first three-dimensional structure of phosphatase 2C. It revealed a new fold in the catalytic core in which two β-sheets form a structure called a β-sandwich surrounded by α-helices. The active site of the enzyme appeared to be on top of the β-sandwich and is composed of two metal ions chelated by several acid residues spread along the sequence of the entire polypeptide (Fig. 3, top panel). It was suggested that metal-bound water coordinates the phosphate group and also serves as a nucleophile for the hydrolytic reaction. The authors also suggested that several other phosphatases, including PDP, should share a similar folding pattern (36). The alignment shown in Fig. 2, made according to Das et al. (36), shows that indeed the secondary structure of the catalytic domain of PDP may be similar to that of PP2C. Both 51- and 56-residue-long insertions within the PDP sequence can be accommodated as loops between helices A1a and A2 and between helix A6 and strand B11, respectively. The alignment also shows that phosphatase 2C contains a carboxyl-terminal domain built of helices A7, A8, and A9, which is absent in the structure of PDP.

The information concerning the sequence alignment and the three-dimensional atomic coordinates from the study of protein phosphatase 2C by Das et al. (36) were used to produce a putative three-dimensional structure of PDP (Fig. 3, middle panel). The coordinates for PP2C were kindly given to us by Dr. David Barford (University of Oxford). The three-dimensional structure of sequence-aligned PDP was obtained by optimization of a molecular probability density function using the program MODELLER, version 4.0 (38). Despite the speculative character of this model, some of the details appear to be quite revealing. First of all, the residues Asn49, Glu53, Asp54, Asp73, Gly74, Asp347, and Asp445 of PDP are likely to form the binucl ear metal center (Asp73, Gly74, Asp347, and Asp445) and to bind the phosphate ion (Asn49, Glu53, and Asp54). This explains why some of the corresponding regions have been previously identified as subdomains of PDP through sequence alignment (21). Second, it appears that the previously mentioned EF-hand motif-like sequence is located within helix A2 and, therefore, that its involvement in the regulation of PDP activity by Ca^{2+} ions is highly unlikely. Third, the 56-residue-long loop 2 (L2), which is absent in the structure of PP2C, appears to be in proximity to the active site (Fig. 3, bottom panel). Therefore, it may shield the active site and require movement in order to make the active site accessible to the protein substrate. This, in turn, should affect the position of Asp445 which is part of the binuclear metal-binding center and, therefore, change the affinity of PDP for the metal ion. It is interesting to note in this respect that the regulation of PDP activity by Ca^{2+} ions, spermine, or the regulatory subunit is associated with changes in the affinity for the metal co-factor that are often accompanied by changes in V_{max} (16, 22). These observations are consistent with the idea that binding of Ca^{2+} ions and/or the regulatory subunit affects the position of L2, making the active site more or less accessible to substrate. Taking into account that part of L2 is involved in chelating Mg^{2+} ions, these move-
ments should simultaneously affect the affinity for the metal co-factor. It is even possible that L2 itself may be a part of the structure responsible for the binding of Ca$^{2+}$ ions because it contains multiple negatively charged residues spread along its entire length.

Enzymatic Activities of PDP1 and PDP2—The physiological role of the isoenzymes of PDP cannot be understood without analysis of their kinetics and regulation. Thus far, these data are available only for isoenzyme PDP1, which has been quite extensively characterized primarily through the efforts of Lester Reed's laboratory (12, 14, 21, 22). The comparable analysis for isoenzyme PDP2 is complicated by the fact that this isoenzyme has never been purified from native sources. Thus, to initiate these studies, we developed an expression system allowing production of the recombinant isoenzymes in E. coli. Both cDNAs were expressed in the BL21(DE3) strain under control of the strong bacteriophage T7 promoter. This system allowed us to purify approximately 10–20 mg of each phosphatase from 1 liter of culture using immobilized metal affinity chromatography. For kinetic experiments, we analyzed the enzymes in E. coli extracts without further purification to avoid interference with phosphatase activity that could be caused by Co$^{2+}$ or Ni$^{2+}$ ions commonly used in immobilized metal affinity chromatography.

![Graphical representation of the alignment of the deduced protein sequences of PDP1, PDP2, and phosphatase 2C.](image)

**Fig. 2.** Alignment of the deduced protein sequences of PDP1, PDP2, and phosphatase 2C. The multiple sequence alignment was made according to Das et al. (36). Residues that are invariant in all three sequences or among PDP sequences are shaded. Residues of PDP that may form the binuclear metal center and bind phosphate ions are indicated by triangles. The elements of the secondary structure of phosphatase 2C are shown by blocks and arrows.
Both isoenzymes appeared to be highly specific for PDC and showed little if any activity for the related mitochondrial multienzyme complex, the branched chain \( \alpha \)-ketoacid dehydrogenase (data not shown). In accord with previous studies (12, 14, 15), the enzymatic activity of PDP1 was stimulated by Ca\(^{2+} \) ions (Fig. 4A). The stimulation by calcium was brought about showing the elements of the catalytic core of PDP. A putative three-dimensional structure of PDP was built using MODELLER, version 4.0 (38). This program models protein three-dimensional structure by satisfying spatial restraints. The input restraints were from the crystal structure of PP2C. The three-dimensional structure of the sequence-aligned PDP was obtained by optimization of a molecular probability density function (38). This procedure uses methods of conjugate gradients and molecular dynamics with simulated annealing. Only regions having residues that can be aligned to the target can be modeled. The output of the program is shown. Invariant residues Asp\(^{73} \), Gly\(^{74} \), Asp\(^{347} \), and Asp\(^{445} \), which may coordinate two magnesium ions, are shown by balls and sticks colored in violet, green, blue, and red, respectively. Regions where loops L1 and L2 of PDP1 should be inserted in the structure of PP2C are shown in red and blue, respectively. Bound phosphate ion is shown by balls and sticks colored red and yellow, respectively. Middle panel, ribbon diagram showing the secondary structural elements of PDP based on the three-dimensional structure of phosphatase 2C. All models are viewed down the edge of the \( \beta \)-sandwich structure. Top panel, ribbon diagram showing the structure of PP2C built based on coordinates provided by Dr. D. Barford. Invariant residues Asp\(^{60} \), Gly\(^{61} \), Asp\(^{239} \), and Asp\(^{282} \), which coordinate two manganese ions, are shown by balls and sticks colored in violet, green, blue, and red, respectively. Regions where loops L1 and L2 of PDP1 should be inserted in the structure of PP2C are shown in red and blue, respectively. Bound phosphate ion is shown by balls and sticks colored red and yellow, respectively. Middle panel, ribbon diagram showing the elements of the catalytic core of PDP. A putative three-dimensional structure of PDP was built using MODELLER, version 4.0 (38). This program models protein three-dimensional structure by satisfying spatial restraints. The input restraints were from the crystal structure of PP2C. The three-dimensional structure of the sequencaligned PDP was obtained by optimization of a molecular probability density function (38). This procedure uses methods of conjugate gradients and molecular dynamics with simulated annealing. Only regions having residues that can be aligned to the target can be modeled. The output of the program is shown. Invariant residues Asp\(^{73} \), Gly\(^{74} \), Asp\(^{347} \), and Asp\(^{445} \), which may coordinate two magnesium ions, are shown by balls and sticks colored in violet, green, blue, and red, respectively. Bottom panel, ribbon diagram showing sizes and positions of loops that should be inserted into the catalytic core of PDP1 (L1 is shown in red; L2 is shown in green). The modeling of loops L1 and L2 are only meant to convey size and general position with respect to the rest of the protein model. Since they are absent in PP2C, there is no target structure to model them with any reliability. This drawing was made using MOLSCRIPT (39).
by an increase in $V_{\text{max}}$ as well as by an increase in sensitivity to $\text{Mg}^{2+}$. In the presence of $\text{Ca}^{2+}$ (total concentration, 0.2 mM), the apparent $K_m$ value of PDP1 for $\text{Mg}^{2+}$ increased approximately 2-fold from 1.5 ± 0.5 to 0.7 ± 0.3 mM, respectively ($n = 5$). Spermine (0.5 mM) has no effect on the activity of PDP1 either in the presence or absence of calcium (data not shown). The enzymatic activity of PDP2 appeared to be comparable with or even higher than that of PDP1 measured in the presence of $\text{Ca}^{2+}$ ions (Fig. 4B). However, this activity could be reached only in the presence of rather high concentrations of $\text{Mg}^{2+}$ (the apparent $K_m$ value of PDP2 for $\text{Mg}^{2+}$ was 17.4 ± 2.7 mM, $n = 5$). Calcium ions up to 0.2 mM had no apparent effect on the activity of PDP2 ($K_m = 16.6 ± 4.5$ mM ($n = 3$) versus 17.4 ± 2.7 mM and $V_{\text{max}} = 94.5 ± 5.5$ pmol/min ($n = 3$) versus 97.5 ± 14.7 pmol/min in the absence or in the presence of calcium, respectively). Surprisingly, the activity of PDP2 appeared to be sensitive to spermine (Fig. 4B). At a concentration of 0.5 mM, it caused a 5-fold increase in the sensitivity of PDP2 to $\text{Mg}^{2+}$ (the apparent $K_m$ value 3.4 ± 1.7 mM, $n = 5$). The increased sensitivity to $\text{Mg}^{2+}$ came, at least to some extent, at the expense of catalytic efficiency. All preparations of PDP2 analyzed in the present study showed a 30–40% decrease in $V_{\text{max}}$ value in the presence of spermine. Thus, the isoenzyme PDP2 appears to be somewhat different with respect to at least some kinetic parameters and regulation. In contrast to PDP1, it is a $\text{Ca}^{2+}$-insensitive enzyme, which can be regulated by spermine. Spermine is unlikely to be a physiologically relevant ligand for PDP2 but rather may mimic the effects of other as yet unknown compounds or of the regulatory subunit. Nevertheless, it is obvious that the physiological role of PDP2 in the regulation of mammalian PDC should be considerably different from that of PDP1.

**Western Blot Analysis of PDP1 and PDP2 in Mitochondria from Skeletal Muscle, Liver, and 3T3-L1 Adipocytes**—Considering the unusual enzymatic properties of PDP2, we were interested in determining the tissues expressing this isoenzyme. Therefore, we analyzed skeletal muscle, where PDC is primarily a catabolic enzyme and is regulated by calcium ions; liver, where PDC is mainly an anabolic enzyme involved in the biosynthesis of lipids; and 3T3-L1 adipocytes, which are a well-established model for studies on the regulation of glycolysis and fatty acid synthesis by insulin. Mitochondria from the above sources were prepared following established protocols and analyzed by Western blotting. Approximately 10 μg of mitochondrial protein was loaded per lane. In studies involving animals, two groups consisting of four animals each were analyzed. In studies with 3T3-L1 cells, we analyzed eight independent preparations of adipocytes. Both anti-PDP1 and anti-PDP2 sera appeared to recognize the immunoreactive protein with a molecular mass of approximately 53 kDa (Fig. 5). However, in muscle mitochondria, it was recognized by anti-PDP1 serum (Fig. 5A, top panel) whereas in liver mitochondria staining was observed mainly with anti-PDP2 serum (Fig. 5B, middle panel). These data, therefore, provide strong evidence that isoenzymes of PDP have a different pattern of tissue distribution. It appears that the $\text{Ca}^{2+}$-sensitive isoenzyme PDP1 is preferentially expressed in muscle mitochondria. In mitochondria from liver, in contrast, it was virtually undetectable, suggesting that if it is expressed there, the level of expression must be very low. On the other hand, isoenzyme PDP2 was present much more abundantly in liver than in muscle. The analysis of mitochondria from 3T3-L1 cells showed abundant expression of isoenzyme PDP2 (Fig. 5B, bottom panel) and only marginal expression, compared with muscle mitochondria, of isoenzyme PDP1 (Fig. 5A, bottom panel). These observations may provide a rationale for previous results, which indicated that PDP in adipose tissue responds to $\text{Ca}^{2+}$ stimulation only at low concentrations of $\text{Mg}^{2+}$ and disappears when the concentration of $\text{Mg}^{2+}$ is raised (25). As shown in the present study, PDP2 has a relatively low activity when the concentration of $\text{Mg}^{2+}$ is low. Thus, it is likely that under these conditions the total PDP activity of adipose tissue primarily reflects the activity of PDP1, which is present in adipose mitochondria at low levels. At high concentrations of $\text{Mg}^{2+}$, when the activity of PDP2 is high, total activity should mainly reflect the contribution of PDP2, which is abundant in adipose mitochondria and is insensitive to $\text{Ca}^{2+}$ ions. It should be noted in this respect that adipose is one of the few tissues where insulin directly regulates the activity of PDP. Taking into account the results of the present study indicating that PDP2 is a major isoenzyme in 3T3-L1 adipocytes, it will be interesting to see the effect of insulin on PDC activity using this model, because such a study may shed new light on the role of PDP2 in the physiological regulation of PDC activity.

The results of the present study provide clear evidence that there are at least two isoenzymes of PDP in mammals that appear to be different with respect to their tissue distribution, kinetic parameters, and regulation. Therefore, it is likely that they are responsible for different aspects of regulation of PDC activity. However, further studies are needed in order to precisely define the contributions made by each of them.

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**REFERENCES**

1. Randle, P. J. (1995) *Proc. Nutr. Soc.* **54**, 317–327
2. Sugden, M. C., Orfali, K. A., and Holness, M. J. (1995) *J. Nutr.* **125**, (suppl.) 1746S–1752S
3. Randle, P. J. (1986) *Biochem. Soc. Trans.* **14**, 799–806
4. Ravindran, S., Radke, G. A., Guest, J. R., and Roche, T. E. (1996) *J. Biol. Chem.* **271**, 653–662
5. Linn, T. C., Pettit, F. H., and Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U. S. A.* **62**, 234–241
6. Huch, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W., and Reed, L. J. (1972) *Arch. Biochem. Biophys.* **151**, 328–340
7. Stepp, L. R., Pettit, F. H., Yeaman, S. J., and Reed, L. J. (1983) *J. Biol. Chem.* **258**, 9454–9458
8. Yeaman, S. J., Hutcheson, E. T., Roche, T. E., Pettit, F. H., Brown, J. R., Reed, L. J., Watson, D. C., and Dixon, G. H. (1978) *Biochemistry* **17**, 2364–2370
9. Sugden, P. H., and Randle, P. J. (1978) *Biochem. J.* **173**, 659–668
10. Korotchkina, L. G., and Patel, M. S. (1995) *J. Biol. Chem.* **270**, 14297–14304
11. Sugden, P. H., Hutson, N. J., Kerbey, A. L., and Randle, P. J. (1978) Biochem. J. 173, 433–435
12. Teague, W. M., Pettit, F. H., Wu, T. L., Silberman, S. R., and Reed, L. J. (1982) Biochemistry 21, 5585–5592
13. Behal, R. H., Buxton, D. B., Robertson, J. G., and Olson, M. S. (1993) Annu. Rev. Nutr. 13, 497–520
14. Pettit, F. H., Roche, T. E., and Reed, L. J. (1972) Biochem. Biophys. Res. Commun. 47, 563–571
15. Severson, D. L., Denton, R. M., Pask, H. T., and Randle, P. J. (1974) Biochem. J. 140, 225–237
16. Rahmatullah, M., and Roche, T. E. (1988) J. Biol. Chem. 263, 8106–8110
17. Chen, G., Wang, L., Liu, S., Chuang, C., and Roche, T. E. (1996) J. Biol. Chem. 271, 28064–28070
18. Gudi, R., Bowker-Kinley, M. M., Kedishvili, N. Y., Zhao, Y., and Popov, K. M. (1995) J. Biol. Chem. 270, 28899–28904
19. Bowker-Kinley, M. M., Davis, W. I., Wu, P., Harris, R. A., and Popov, K. M. (1996) Biochem. J. 329, 191–196
20. Rowles, J., Scherer, S. W., Xie, T., Majer, M., Nickel, D. C., Rommens, J. M., Popov, K. M., Harris, R. A., Riebow, N. L., Xia, J., Tsui, L. C., Bogardus, C., and Roche, T. E. (1996) J. Biol. Chem. 271, 22376–22382
21. Lawson, J. E., Niu, X. D., Browning, K. S., Trong, H. L., Yan, J., and Reed, L. J. (1993) Biochemistry 7, 8987–8993
22. Yan, J., Lawson, J. E., and Reed, L. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 14, 4953–4956
23. Song, H., and Komuniecki, R. (1994) J. Biol. Chem. 269, 31573–31578
24. De Burgos, N. M. G., Gallina, F., Burgos, C., and Blanco, A. (1994) Arch. Biochem. Biophys. 306, 520–524
25. Rutter, G. A., McCormack, J. G., Midgley, P. J., and Denton, R. M. (1989) Annu. N.Y. Acad. Sci. 573, 206–217
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, pp. 8.46–8.47, 17.3–17.41, and 18.40–18.41, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Chisholm, D. (1989) BioTechniques 7, 21–23
28. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 53–138, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Wu, P., Sato, J., Zhao, Y., Jaskiewicz, J., Popov, K. M., and Harris, R. A. (1996) Biochem. J. 329, 197–201
30. Laemmli, U. K. (1970) Nature 227, 680–685
31. Kedishvili, N. Y., Popov, K. M., Jaskiewicz, J., and Harris, R. A. (1994) Arch. Biochem. Biophys. 315, 317–322
32. Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T., and Denton, R. M. (1976) Biochem. J. 154, 327–348
33. Johnson, D., and Lardy, H. (1967) Methods Enzymol. 10, 94–96
34. McCormack, J. G., and Denton, R. M. (1980) Biochem. J. 190, 95–105
35. Lowry, O. H., Rosebrough, N. H., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
36. Das, A. K., Helps, N. R., Cohen, P. T. W., and Barford, D. (1996) EMBO J. 15, 6798–6809
37. Von Heijne, G. (1986) EMBO J. 5, 1351–1355
38. Šali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
39. Kraulis, P. (1991) J. Appl. Crystallogr. 24, 946–950