Cloning, Expression, and Properties of the Regulatory Subunit of Bovine Pyruvate Dehydrogenase Phosphatase

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as described elsewhere (15). Double-stranded DNA was sequenced with the Sequenase V 2.0 kit from U. S. Biochemical Corp. (Amersham Corp.). Both strands of each fragment were sequenced completely. Multiple clones were sequenced to minimize potential PCR artifacts.

Sequence Analysis—Beckman Microgenie version 6 was used to analyze DNA and amino acid sequence data. The BLAST program (16) was used to compare sequences with GenBank™, Protein Information Resource Data Base, Brookhaven Protein Data Base, Kabat Sequences of Proteins, and Swiss Data Bases. MACAW (17) was used to optimize sequence alignments.

Expression and Purification of Recombinant MBP-PDPr Fusion—Oligonucleotide primers R99 and R104, respectively, introduced an EcoRI site to the 5' end and a SalI site to the 3' end of the 2.5-kilobase pair fragment encoding the mature PDPr. The fragment was subcloned into the EcoRI and SalI sites of pMAL-C2 to produce plasmid pR99-12. This plasmid was transformed into E. coli strain BL21(DE3). Colonies containing the plasmid were selected by growth on LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract) containing ampicillin (50 μg/ml). A 45-ml stationary phase culture grown on the same medium containing 2% glucose was used to inoculate 1.5 liter of LB medium containing 100 μg/ml ampicillin and 2 μg/ml benzoylexycarbonyl-Phe-Ala-CHN₂, and 0.1% Triton X-100. All operations were carried out at about 4 °C. The cells were disrupted by ultrasonic treatment using five 15-s pulses at 60 W, alternating with 1-min intervals to permit cooling. The suspension was diluted to 60 ml with buffer B (buffer A lacking Triton X-100) and centrifuged at 17,000 g, for 30 min. The extract was applied to an amyllose resin column (2.5 × 3 cm) that had been equilibrated with buffer B. The column was washed with 300 ml of buffer B and then eluted with 10 ml of buffer C containing 10 mM maltose. The fractions containing the MBP-PDPr fusion, detected by SDS-PAGE and immunoblotting, were pooled and diluted 5-fold with buffer C (buffer B lacking NaCl). The solution was applied to a heparin-agarose column (2.5 × 5 cm) equilibrated with buffer D (20 mM MOPS buffer, pH 7.4, 50 mM NaCl, 10 μM FAD, 10 mM 2-mercaptoethanol, and 10 μM EDTA, 1 mM phenylmethanesulfonyl fluoride, 2 mM benzamidine, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 2 μg/ml benzyl-oxycarbonyl-Phe-Ala-CHN₂, and 0.1% Triton X-100). Operations were carried out at about 4 °C. The cells were disrupted by ultrasonic treatment using five 15-s pulses at 60 W, alternating with 1-min intervals to permit cooling. The suspension was diluted to 60 ml with buffer B (buffer A lacking Triton X-100) and centrifuged at 17,000 × g for 30 min. The extract was applied to an amylose resin column (2.5 × 3 cm) that had been equilibrated with buffer B. The column was washed with 300 ml of buffer B and then eluted with 10 ml of buffer C containing 10 mM maltose. The fractions containing the MBP-PDPr fusion, detected by SDS-PAGE and immunoblotting, were pooled and diluted 5-fold with buffer C (buffer B lacking NaCl). The solution was applied to a heparin-agarose column (2.5 × 5 cm) equilibrated with buffer D (20 mM MOPS buffer, pH 7.4, 10 mM 2-mercaptoethanol, and 10 μM FAD). The column was washed with 60 ml of buffer D and then developed with a 100-ml linear gradient from 0.0 to 0.5 mM NaCl in buffer D. The fractions containing the fusion protein were pooled and concentrated in a Centri-30 concentrator. The yield of highly purified MBP-PDPr was 5–6 mg.

Cleavage of MBP-PDPr and Purification of PDPr—For cleavage of MBP-PDPr, the solution was dialyzed against buffer D containing 50 mM NaCl and then incubated with factor Xa (approximately 2% by weight of fusion protein) for 20 h at 4 °C in the presence of 1 mM CaCl₂. MBP and uncleaved fusion protein were removed by chromatography on Q-Sepharose and amylose resin. The incubation mixture was dialyzed against 20 mM MOPS, pH 8.0, 10 mM 2-mercaptoethanol, 10 μM FAD, and protease inhibitors. The solution was applied to a Q-Sepharose column (1.0 × 4.0 cm) that had been equilibrated with the same buffer. The column was washed with three column volumes of the buffer.

Immunoblotting—Proteins were separated by SDS-PAGE on 12.5% gels and transferred electrophoretically to Immobilon-P membrane (Millipore). Blots were probed with a 1:4000 dilution of rabbit anti-PDPr IgG or anti-PDPc IgG followed by detection with a 1:4000 dilution of goat anti-rabbit IgG (Bio-Rad). Rabbit antibodies to PDPr and PDPc were purified on protein A-agarose (Sigma) to obtain the IgG fraction.

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and then developed with a linear gradient from 0.0 to 0.5 M NaCl. The fractions containing PDPr were pooled, diluted 1:3 with 20 mM MOPS, pH 7.4, 50 mM NaCl, 10 mM FAD and protease inhibitors and applied to an amylose resin column (1.0 x 3.0 cm) that had been equilibrated with the same buffer. The flow-through fractions containing PDPr were pooled and concentrated in a Centricon-30 concentrator.

RESULTS AND DISCUSSION

Isolation and Characterization of cDNA Fragments—Attempts to isolate clones encoding PDPr from bovine cDNA libraries were unsuccessful. A PCR-based approach eventually proved to be successful. Degenerate oligonucleotide primers were synthesized based on experimentally determined amino acid sequences of native PDPr (underlined in Fig. 2). Combinations of the forward and reverse degenerate primers were used in PCR with bovine genomic DNA as the template. Using primers R23 and R24 (Table I), based on a 30-amino acid residue sequence (residues 414–443), DNA fragment R71-2 was amplified. The sequence of R71-2, encoding residues 417–439 of PDPr, was used to design specific oligonucleotide primers. Whenever possible, poly(A)+ RNA was used as the template for reverse transcriptase in 3′ and 5′ RACE. Total RNA was used when the use of poly(A)+ RNA was not successful.

Specific nested forward primers R26 and R27 were used in 3′ RACE with bovine total RNA as the template to produce fragment 3R-3. This fragment contains the cDNA encoding residues 429–503, followed by 155 bp of intron DNA (I-2, sequence not shown). Successive 3′ RACE using specific primers based on 3R-3 were unsuccessful in extending the cDNA. Therefore, inverse PCR was used to obtain fragment 17-4, encoding amino acid residues 504–590. Bovine genomic DNA was digested into linear fragments with ApaI. Each fragment was ligated to itself to form a circular molecule. Specific primers R39, a reverse primer based on the sequence of an intron (I-1, see below), and R33, a forward primer based on the sequence of 3R-3, were used to amplify the DNA around the circle. Primers based on

FIG. 2. Nucleotide sequence of PDPr cDNA and the deduced amino acid sequence (one-letter amino acid symbols). The experimentally determined amino acid sequences are underlined with a solid line. A putative FAD-binding motif is underlined with a dashed line.

FIG. 3. SDS-PAGE and immunoblot analyses of purified recombinant MBP-PDPr and PDPr. A, SDS-PAGE patterns; the gel was stained with Coomassie Brilliant Blue; lane 1, protein standards; lane 2, cell-free extract of induced E. coli; lane 3, fraction purified by affinity chromatography; lane 4, fraction purified by heparin-agarose chromatography; lane 5, after treatment of purified MBP-PDPr with Factor Xa; lane 6, MBP in sample 5 after removal of MBP by chromatography. Approximately 7, 9.8, 4, 1.2, 5.5, and 4.5 µg of protein were applied to lanes 1–6, respectively. The protein standards were myosin, β-galactosidase, phosphorylase b, bovine serum albumin, glutamate dehydrogenase, lactate dehydrogenase, and carbonic anhydrase. Molecular weights are shown as Mr 31023; lane 1, immunoblot analysis of purified MBP-PDPr (1.8 µg) using anti-PDPr IgG; lane 2, PDPr (1.3 µg) after release and removal of MBP.

and then developed with a linear gradient from 0.0 to 0.5 mM NaCl. The fractions containing PDPr were pooled, diluted 1:3 with 20 mM MOPS, pH 7.4, 50 mM NaCl, 10 µM FAD and protease inhibitors and applied to an amylose resin column (1.0 x 3.0 cm) that had been equilibrated with the same buffer. The flow-through fractions containing PDPr were pooled and concentrated in a Centricon-30 concentrator.

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the sequence of 17-4 were used in 3′ RACE with poly(A)⁺ RNA as the template. Fragments 3R-5f and 3R-9-7, encoding amino acid residues 559–695 and 697–847, respectively, and the 3′-flanking DNA, were amplified. Possibly due to the secondary structure of the mRNA, attempts to use 3′ RACE to amplify the cDNA between fragments 3R-5f and 3R-9-7 were unsuccessful. The missing region was synthesized by PCR with a forward primer, R79, based on 3R-5f and a reverse primer, R82, based on the sequence of human cDNA clone R09107, and cDNA transcribed from poly(A)⁺ RNA. The deduced amino acid sequence of the overlapping human cDNA clones R15456, R17108, R09106, and R09107 (GenBank™ accession nos.) from the Expressed Sequence Tag Data Base (dbEST) was found to be approximately 92% identical to amino acid residues 449–766 of PDPr and was helpful in determining the intron-exon boundaries.

5′ Race was employed to obtain sequences upstream of fragment R71-2. 5′ RACE using poly(A)⁺ RNA was not successful; therefore, the upstream regions of the cDNA were obtained using total RNA as the template for reverse transcriptase. Nested reverse primers R29 and R32, based on the sequences of fragments R71-2 and 3R-3, respectively, were used to amplify fragment 5R-48. This fragment terminated prematurely downstream of the 5′ end of the full-length cDNA, but its sequence was used to design additional nested primers. The remainder of the 5′ portion of the cDNA was contained in 5R-305, 5R-432, and 5R-512, overlapping fragments amplified in consecutive rounds of 5′ RACE using primers based on the sequence obtained in the preceding round. A 1633-bp intron was found beginning at nucleotide 1463 (sequence not shown). The splice site is between amino acids 20 and 21 of one of the experimentally determined sequences (amino acid residues 388–411).

Both the 5′ and 3′ ends of the two introns are consistent with the consensus sequences for mammalian introns (18). The molecular basis of the presence of introns in the cDNA for PDPr remains to be determined.

Reconstruction of the Full-length cDNA—The continuous sequence of the full-length cDNA was compiled from the fragments diagrammed in Fig. 1. Specific primers based on the cDNA sequence were used to generate R99-90, R70-100, and R51-75, three large overlapping PCR fragments from cDNA transcribed from poly(A)⁺ RNA. Primers R99 and R100 were used to amplify fragment R99-100, encoding the mature PDPr, from the three overlapping fragments. These primers introduced an EcoRI site to the 5′ end and a HindIII site to the 3′ end, respectively. Fragment R99-100 was subcloned into pCRII. Both strands of multiple clones were sequenced. The cDNA is 2885 bp in length and contains an open reading frame of 2634 bp encoding a putative mitochondrial presequence of 31 amino acids.

### Table II

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<th>Assay mixtures</th>
<th>Phosphatase activity</th>
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<td>PDPc</td>
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<tr>
<td>PDPc + MBP-PDPr</td>
<td>0.037</td>
</tr>
<tr>
<td>PDPc + MBP-PDPr + spermine</td>
<td>0.061</td>
</tr>
<tr>
<td>PDPc + spermine</td>
<td>0.064</td>
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The assay mixtures contained 0.5 μg of PDPc, 0.5 mM MgCl₂, 0.1 mM CaCl₂, 0.1% 2-mercaptoethanol, phosphorylated pyruvate dehydrogenase complex containing 0.14 nmol of ³²P-labeled protein-bound phosphate groups (~24,000 cpm), 25 mM MOPS buffer, pH 7.3, and, where indicated, 3 μg of MBP-PDPr fusion, and 1.0 mM spermine in a total volume of 40 μl.

Activity is expressed as nmol of ³²P, released per min.

**FIG. 4.** Analysis by polyacrylamide gel electrophoresis of binding of MBP-PDPr and PDPr to PDPc. The samples were subjected to PAGE in the absence of SDS, and the gel was stained with Coomassie Brilliant Blue; lane 1, native PDP (4.8 μg); lane 2, recombinant PD (1.2 μg); lane 3, mixture of recombinant PDP (1.2 μg) and MBP-PDPr (2 μg); lane 4, mixture of recombinant PDP (2 μg) and PDPr (3.7 μg).

**FIG. 5.** Alignment of amino acid sequences (single-letter code) of bovine PDPr and rat liver dimethylglycine dehydrogenase. The deduced sequences were aligned for maximum similarity using MACAW. Identical residues are marked with a black dot (●).
amino acid residues and a mature protein of 847 residues with a calculated molecular weight of 95,656. The deduced amino acid sequence of PDPr contains the nine experimentally determined peptide sequences (Fig. 2). Furthermore, the calculated $M_r$ of PDPr is in agreement with the molecular mass of native PDPr (95,800 ± 200 Da) determined by MALDI mass spectrometry. A putative consensus FAD-binding motif (GXGXXG) is located near the amino terminus (residues 17–22).

Expression, Purification, and Properties of Recombinant PDPr—Several attempts to overexpress PDPr in *E. coli* produced largely insoluble protein. Soluble PDPr was expressed as a fusion with *E. coli* maltose-binding protein. When analyzed by SDS-PAGE and by immunoblotting (Fig. 3), the purified MBP-PDPr showed a major band that migrated as expected for the fusion protein with a calculated molecular weight of 138,151, and it reacted with anti-native PDPr IgG. Like native PDPr (5), MBP-PDPr decreased the sensitivity of PDPc to Mg$^{2+}$, and this inhibition was reversed by the polyamine spermine (Table II). Spermine had little effect, if any, on the sensitivity of PDPc to Mg$^{2+}$ (Table II) (5). We interpret these data to indicate that spermine interacts with PDPr. The observation that spermine does not cause PDPr to dissociate from PDPc$^2$ indicates that spermine produces a conformational change in PDPr (i.e. an allosteric effect) that reverses its inhibitory effect on PDPc. Unlike native PDPr, MBP-PDPr does not bind tightly to PDPc (Fig. 4). However, after treatment of MBP-PDPr with factor Xa to release MBP (Fig. 3), the PDPr did bind PDPc (Fig. 4). Apparently, the MBP component sterically inhibits tight binding of MBP-PDPr to PDPc. These results demonstrate that the recombinant PDPr possesses properties characteristic of the native PDPr. Availability of cloned cDNA for PDPr should facilitate further studies on structure-function relationships in PDPr.

Comparison of Amino Acid Sequences of PDPr and Dimethylglycine Dehydrogenase—A BLAST search of protein data bases revealed 32% sequence identity between bovine PDPr and the rat liver flavoprotein, dimethylglycine dehydrogenase (19) (Fig. 5). The overall pattern indicates that the two proteins evolved from a common ancestor. Dimethylglycine dehydrogenase is a participant in the choline degradative pathway, is localized to the mitochondrial matrix, has a calculated $M_r$ of 91,600, and contains covalently bound FAD (20). Because of this apparent evolutionary relationship and the fact that PDPr is a flavoprotein, the possibility was considered that PDPr possesses a specific dehydrogenase activity and that this activity may be involved in its regulatory function. However, PDPr did not exhibit activity with dimethylglycine or other members of the choline degradative pathway (choline, betaine, and sarcosine) in an assay coupled to phenazine methosulfate and 2,6-dichloroindophenol (20) (data not shown). Antiserum to rat liver dimethylglycine dehydrogenase did not react with bovine PDPr and antiserum to PDPr did not react with dimethylglycine dehydrogenase (data not shown).

The role, if any, of the dissociable FAD in PDPr remains to be determined. It is possible that PDPr has a redox-sensitive function. Another possibility is that the FAD has a structural role. A few enzymes require FAD for catalytic activity, although no redox reaction is involved, e.g. acetohydroxy acid synthases (21). The availability of recombinant PDPr should facilitate studies on the role of FAD in PDPr.

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REFERENCES

—S. H. Park and L. J. Reed, unpublished data.