Nematode pyruvate dehydrogenase kinases: role of the C-terminus in binding to the dihydrolipoyl transacetylase core of the pyruvate dehydrogenase complex

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Pyruvate dehydrogenase kinases (PDKs) from the anaerobic parasitic nematode *Ascaris suum* and the free-living nematode *Caenorhabditis elegans* were functionally expressed with hexahistidine tags at their N-termini and purified to apparent homogeneity. Both recombinant PDKs (rPDKs) were dimers, were not autophosphorylated and exhibited similar specific activities with the *A. suum* pyruvate dehydrogenase (E1) as substrate. In addition, the activities of both PDKs were activated by incubation with PDK-depleted *A. suum* muscle pyruvate dehydrogenase complex (PDC) and were stimulated by NADH and acetyl-CoA. However, the recombinant *A. suum* PDK (rAPDK) required higher NADH/NAD^+ ratios for half-maximal stimulation than the recombinant *C. elegans* PDK (rCPDK) or values reported for mammalian PDKs, as might be predicted by the more reduced microaerobic mitochondrial environment of the APDK. Limited tryptic digestion of both rPDKs yielded stable fragments truncated at the C-termini (trPDKs). The trPDKs retained their dimeric structure and exhibited substantial PDK activity with the *A. suum* E1 as substrate, but PDK activity was not activated by incubation with PDK-depleted *A. suum* PDC or stimulated by elevated NADH/NAD^+ or acetyl-CoA/CoA ratios. Direct-binding assays demonstrated that increasing amounts of rCPDK bound to the *A. suum* PDK-depleted PDC. No additional rCPDK binding was observed at ratios greater than 20 mol of rCPDK/mol of PDC. In contrast, the truncated rCPDK (trCPDK) did not exhibit significant binding to the PDC. Similarly, a truncated form of rCPDK, $rCPDK_{1–334}$, generated by mutagenesis, exhibited properties similar to those observed for trCPDK. These results suggest that the C-terminus of the PDK is not required for subunit association of the homodimer or catalysis, but instead seems to be involved in the binding of the PDKs to the dihydrolipoyl transacetylase core of the complex.

Key words: *Ascaris suum*, *Caenorhabditis elegans*, E2 binding domain, nematode, pyruvate dehydrogenase kinase.

INTRODUCTION

The pyruvate dehydrogenase complex (PDC) catalyses the oxidative decarboxylation of pyruvate, the rate-limiting step in the aerobic oxidation of pyruvate [1]. The PDC is a large multienzyme complex consisting of three catalytic components: pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2) and dihydrolipoyl dehydrogenase (E3) plus an E3-binding protein, formerly known as protein X [1,2]. PDC activity in higher eukaryotes is regulated by reversible phosphorylation/dephosphorylation catalysed by a distinct E1 kinase (PDK) and E1 phosphatase [2,3]. The PDK co-purifies with the complex and is tightly associated with the inner lipoyl domain of the E2 core [4]. However, the E2-binding domain of the PDK has not yet been characterized.

Multiple mammalian PDK isoforms have been identified that seem to have unique tissue distributions and physiological roles [5–9]. Interestingly, although these PDK isoforms phosphorylate serine residues, they lack motifs usually present in eukaryotic Ser/Thr kinases and exhibit significant sequence identity with histidine protein kinases best characterized in prokaryotic signal transduction [6,7,10,11]. PDK activity is enhanced by binding to the E2 core and can be modulated by intramitochondrial ratios of NADH to NAD^+ and of acetyl-CoA to CoA [9,12]. The effects of NADH and acetyl-CoA on PDK activity are mediated by the degree of E3-catalysed reduction and E2-catalysed acetylation of the inner lipoyl domain of E2 [13–15], and each PDK isoform seems to exhibit a different sensitivity to NADH and acetyl-CoA [9].

The PDC has a key role in the anaerobic mitochondrial metabolism of the parasitic nematode *Ascaris suum* and is designed to function under the elevated intramitochondrial NADH/NAD^+ and acetyl-CoA/CoA ratios present during anaerobiosis [16,17]. For example, overall PDC activity is less sensitive to NADH inhibition and the PDK requires higher NADH/NAD^+ ratios for maximal stimulation [18,19]. Therefore to improve our understanding of the regulation of PDC activity during the aerobic-to-anaerobic transition in *A. suum* development and, more specifically, the role of the E2 core and E3 in mediating NADH sensitivity, PDKs from anaerobic *A. suum* muscle (APDK) and the aerobic free-living nematode *Caenorhabditis elegans* (CPDK) were functionally expressed in *Escherichia coli* and purified to homogeneity ([20], and the present study). Neither of the recombinant nematode PDKs was autophosphorylated, in contrast with results reported for mammalian PDKs. The activities of both nematode rPDKs were stimulated by binding to PDK-depleted *A. suum* PDC and elevated ratios of NADH to NAD^+ and of acetyl-CoA to CoA.

Abbreviations used: APDK, *Ascaris suum* pyruvate dehydrogenase kinase; CPDK, *Caenorhabditis elegans* pyruvate dehydrogenase kinase; E1, pyruvate dehydrogenase; E2, dihydrolipoyl transacetylase; E3, dihydrolipoyl dehydrogenase; MBP, maltose-binding protein; MBP–$rCPDK_{284–402}$, $rCPDK_{284–402}$ attached at the C-terminus of maltose-binding protein; Ni-NTA-agarose, Ni-nitrilotriacetic acid–agarose; p45, 45 kDa component of *A. suum* PDC; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; rAPDK, recombinant APDK; rCPDK, recombinant CPDK; $rCPDK_{1–334}$, a truncated rCPDK containing residues 1–334; $rCPDK_{284–402}$, C-terminus of rCPDK containing residues 284–402; trAPDK, 37 kDa fragment of rAPDK obtained by limited tryptic digestion; trCPDK, 38 kDa fragment of rCPDK obtained by limited tryptic digestion.

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Tryptic digestion of both recombinant rPDKs yielded stable truncated forms of the enzymes, which retained PDK activity with E1 as substrate but were not bound to PDK-depleted PDC or stimulated by elevated NADH/NAD^+ ratios. These results suggest that a region near the C-terminus of the PDK is responsible for binding to the PDC.

EXPERIMENTAL

Materials

Adult *A. suum* were collected from pig intestines at Routh Packing (Sandusky, OH, U.S.A.). PDC (3–5 μmol of NADH/min per mg of protein) was isolated from frozen *A. suum* muscle strips, and native *A. suum* E1 from purified adult *A. suum* muscle PDC, as described previously [18,21]. PDK-depleted PDC was prepared as described previously [20]. [γ-^{32}P]ATP was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). Restriction enzymes, amylose resin and maltose-binding protein (MBP) were purchased from New England Biolabs. Sequencing-grade trypsin was obtained from Promega. Ni-nitrilotriacetic acid–agarose (Ni-NTA-agarose) was obtained from Qiagen. All electrophoresis reagents were from Bio-Rad. All other chemicals and reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Cloning and sequencing of CPDK

A putative CPDK sequence, ZK370.5, was identified in the *C. elegans* genome database [22]. To clone the full-length CPDK cDNA, a gene-specific primer CPDK3-5 (5′-TGATTGAGCTGGACGATTTCCTTGACC-3′), corresponding to bases 19684–19710 of cosmid ZK370, and a λgt11 forward primer (5′-GGTGGCGACGACTCCTGGAGCCCG-3′) were used for reverse transcriptase-mediated PCR with a *C. elegans* λgt11 cDNA library as a template (10^8 plaque-forming units/ml) [kindly provided by Dr. Wanyuan Ao (University of Alberta, Edmonton, Alberta, Canada)]. Before use, the λgt11 cDNA library (7 μl) was heated at 70 °C for 5 min, then placed on ice. The 1.2 kb reverse transcriptase-mediated PCR product was purified and subcloned into the pCR II vector (InVitrogen). Plasmid DNA was sequenced by the dideoxy chain termination method [23] with Sequenase version 2.0 (U.S. Biochemicals Corp.).

Expression of APDK, CPDK, C-terminal truncated forms of recombinant CPDK (rCPDK) and MBP–rCPDK$_{284–402}$ in *E. coli*

APDK was expressed in *E. coli* as a fusion protein with a hexahistidine tag at its N-terminus, as described previously [20]. CPDK was expressed in *E. coli* as a fusion protein with a His_6 tag at its N-terminus by using a similar protocol [24]. In brief, *Bam*HI and *Pst*I restriction sites flanking the sequence coding for the mature CPDK (without the first nine N-terminal residues) were constructed by PCR. The resulting *Bam*HI/*Pst*I fragment was then ligated into pQE-30 cut with *Bam*HI and *Pst*I and the plasmid was expressed in *E. coli* M15 (pREP4). C-terminal truncated forms of rCPDK were generated by the insertion of a stop codon at designated positions by using a Quikchange site-directed mutagenesis kit (Stratagene). To attach the C-terminus of CPDK (rCPDK$_{284–402}$, from residues 284 to 402 plus the stop codon) to the C-terminus of MBP, *Bam*HI and *Pst*I restriction sites flanking the sequence coding for rCPDK$_{284–402}$ were constructed by PCR. The resulting 370 bp *Bam*HI/*Pst*I fragment then was ligated into the pMAL-c2 vector (New England Biolabs) downstream of the *malE* gene, which encodes MBP, to generate the MBP–rCPDK$_{284–402}$ fusion protein. The plasmid was expressed in *E. coli* JM109 (Promega).

Purification of recombinant APDK (rAPDK), rCPDK and MBP–rCPDK$_{284–402}$

rAPDK was purified as described previously [20]. *E. coli* expressing rCPDK or MBP–rCPDK$_{284–402}$ were grown at room temperature in 1 litre of tryptone/phosphate media [2 % (w/v) Bacto-tryptone/0.2 % Na_2HPO_4/0.1 % KH_2PO_4/0.8 % NaCl/1.5 % (w/v) yeast extract] until a D_{600} of 0.6 was reached. Then isopropyl β-D-thiogalactoside was added to a final concentration of 0.4 or 0.1 mM respectively, and induction continued overnight at 20 °C with vigorous shaking. Bacteria were collected by centrifugation. To purify rCPDK, the pellet (approx. 10 g) was resuspended in 30 ml of 50 mM NaH_2PO_4 (pH 8.0)/5 mM 2-mercaptoethanol/0.5 % (v/v) Triton X-100/300 mM NaCl (buffer I), sonicated on ice and centrifuged at 20000 ***g*** for 20 min. Imidazole was added to the supernatant to a final concentration of 40 mM and the supernatant was mixed for 60 min with a slurry of Ni-NTA-agarose previously equilibrated in buffer I. Then the resin was loaded into a column, washed with 50 mM Na_2HPO_4 (pH 6.0)/5 mM 2-mercaptoethanol/100 mM imidazole/300 mM NaCl (buffer II) and eluted with a 60 ml linear gradient of 0.1–0.5 M imidazole in buffer II. Purified rCPDK was dialysed against 50 mM Mops (pH 7.4)/1 mM $MgCl_2$/1 mM dithiothreitol/300 mM KCl, concentrated by ultrafiltration (Centriprep 10; Amicon) and stored in aliquots in 20 % (v/v) glycerol at −70 °C until use.

To purify MBP–rCPDK$_{284–402}$, the bacterial pellet (4 g) was resuspended in 30 ml of 50 mM Mops (pH 7.4)/1 mM $MgCl_2$/1 mM EDTA/300 mM NaCl/1 mM PMSF/1 mM benzamidine/1 μg/ml leupeptin (buffer III), sonicated on ice and centrifuged at 20000 ***g*** for 20 min. The supernatant was diluted (1:5) with buffer III and loaded on an amylose resin column. The column was washed with buffer III and protein was eluted with 10 mM maltose in buffer III. Purified MBP–rCPDK$_{284–402}$ was concentrated by ultrafiltration (Centriprep 10; Amicon) and stored in aliquots in 20 % (v/v) glycerol at −70 °C until use.

Limited tryptic digestion of rCPDK and rAPDK

Purified rCPDK (10 mg/ml) or rAPDK (7.6 mg/ml) was diluted to 1 mg/ml in 50 mM Mops (pH 7.4)/1 mM $MgCl_2$/1 mM dithiothreitol/300 mM NaCl. Modified trypsin was added to a final protease-to-protein ratio of 1:20 (w/w). Digestion was performed at 37 °C for 5, 10 or 15 min. Proteolysis was stopped by either the addition of excess trypsin inhibitor for the assay of PDK activity or the addition of 2 × SDS sample buffer and boiling for 10 min for SDS/PAGE.

Binding of the 38 kDa fragment of rCPDK obtained by limited tryptic digestion (trCPDK), rCPDK and MBP–rCPDK$_{284–402}$ to PDK-depleted PDC

PDK-depleted PDC (300 μg) was incubated with rCPDK, trCPDK, MBP–rCPDK$_{284–402}$ or MBP at 22 °C in 20 mM Tris/HCl (pH 7.4)/5 mM $MgCl_2$/50 mM KCl/5 mM dithiothreitol (buffer IV) in a total volume of 250 μl. After 15 min, the incubation mixtures were layered over a three-step sucrose gradient [1 ml steps of 7.5 %, 10 % and 15 % (w/v)] in 50 mM Mops (pH 7.4)/1 mM $MgCl_2$/0.1 mM EDTA/3 mM dithiothreitol (buffer V) and centrifuged at 155000 ***g*** for 3 h. After centrifugation, the pellets were resuspended in buffer V. Samples were assayed for PDK activity or analysed by either SDS/PAGE or immunoblotting.

Assay of PDK activity

To measure the initial rate of incorporation of ^{32}P from [γ-^{32}P]ATP, fractions were preincubated with either native *A. suum* E1 or adult *A. suum* APDK-depleted muscle PDC for 1 min at 37 °C in buffer IV. The reaction was initiated with the addition of Mg[γ-^{32}P]ATP^{2-} (2 mM $MgCl_2$/0.5 mM ATP, final concentration; 150 c.p.m./pmol). After 15, 30, 45, 60 and 90 s incubation, aliquots were spotted on 2 cm × 2 cm Whatman P81 filter paper, washed three times with agitation in 75 mM phosphoric acid, dried and counted for protein-bound radioactivity.

Gel electrophoresis and determination of N-terminal sequence and apparent molecular masses of native proteins

SDS/PAGE was performed by the method of Laemmli [25] and proteins were revealed with 0.1 % Coomassie Brilliant Blue R-250. Autoradiographs were obtained after exposure of Kodak X-Omat X-ray film to the vacuum-dried gels for 6 h at −70 °C. Determination of the N-terminal sequence was performed as described previously [21]. The apparent molecular masses of native rCPDK, trCPDK and the 37 kDa fragment of rAPDK obtained by limited tryptic digestion (trAPDK) were determined by chromatography on a Superose 12 column equilibrated in 50 mM Mops (pH 7.4)/1 mM $MgCl_2$/1 mM dithiothreitol/300 mM KCl. The flow rate was 0.5 ml/min.

Preparation of antibody and immunoblotting

Purified rCPDK was separated by SDS/PAGE [12 % (w/v) gel], stained with Coomassie Blue, and used to immunize rabbits as described previously [26]. Antibody against rCPDK was affinity-purified by coupling rCPDK to CNBr-activated Sepharose 4B (Sigma) by the method of Kent [27]. For immunoblotting, samples were separated by SDS/PAGE [10 % (w/v) gel], transferred to nitrocellulose (Schleicher & Schuell) overnight at 250 mA in 25 mM Tris/HCl/192 mM glycine/20 % (v/v) methanol. The membrane was blocked with 1 % (w/v) BSA. Immunoblotting used a goat anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase as the secondary antibody and the Protoblot Western blot AP system (Promega Corp.). The immunoblots were developed with Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate.

RESULTS

Expression of rAPDK and rCPDK

The putative mature rCPDK was expressed in *E. coli* with a His_6 tag at its N-terminus and purified to apparent homogeneity by chromatography on Ni-NTA-agarose, as we have described recently for the expression of rAPDK [20]. Yields of purified rCPDK ranged from 5 to 12 mg/l of bacterial culture, and the co-expression of GroES and GroEL was not necessary to obtain substantial amounts of protein in the soluble fraction. Purified rCPDK migrated as a single, sharp band during SDS/PAGE with an apparent molecular mass of approx. 45 kDa (Figure 1, lane 1). In contrast, native rCPDK migrated with an apparent molecular mass of approx. 76 kDa during FPLC on Superose 12, suggesting that the native rCPDK was a dimer, as has been observed for PDKs from other sources [2,20].

Regulation of rCPDK activity

Purified rCPDK and rAPDK exhibited PDK activity with both the free *A. suum* E1 and E1 bound to PDK-depleted preparations of *A. suum* muscle PDC (Tables 1 and 2). More importantly, the addition of PDK-depleted *A. suum* PDC markedly stimulated the PDK activity of both rCPDK (94 ± 9 compared with 309 ± 28 nmol of ^{32}P incorporated/min per mg of protein) and rAPDK (98 ± 13 compared with 365 ± 10 nmol of ^{32}P incorporated/min per mg of protein) (Table 2), presumably by the binding of the rPDKs to the E2 core of the complex. These results are in agreement with those reported for mammalian PDKs and suggest that the domains responsible for the binding of the nematode PDKs to the lipoyl domain of E2 are conserved, because both rAPDK and rCPDK bound the *A. suum* PDC. Previous workers also have demonstrated that PDKs are sensitive to the E3-catalysed reduction and E2-catalysed acetylation of the lipoyl groups of E2 [13–15]. Therefore to examine the effects of NADH and acetyl-CoA on the activity of the nematode rPDKs, the rPDKs were incubated with PDK-depleted PDC in NADH/NAD^+ (1:200 or 3:1 molar ratios) or NADH/NAD^+ plus acetyl-CoA and then assayed for PDK activity, as described recently for the characterization of the mammalian PDK isoforms [9]. As indicated in Table 1, elevated NADH/NAD^+ ratios stimulated the activity of both rPDKs; the addition of acetyl-CoA further enhanced this stimulation (Table 1). The maximal stimulation of rAPDK was greater than that of rCPDK (Table 1). However, rCPDK appeared to be more sensitive to increasing NADH/NAD^+ ratios. When the NADH/NAD^+ ratio was varied at a fixed nucleotide concentration, the half-maximal stimulation of PDK activity occurred at a ratio of 0.21 ± 0.03 for rAPDK and 0.06 ± 0.01 for rCPDK (results not shown).

Autoradiography of SDS/polyacrylamide gels of rCPDK incubated with [^{32}P]ATP in the presence and in the absence of native *A. suum* E1 revealed no evidence for autophosphorylation (Figure 2), in contrast with results reported for recombinant mammalian PDKs [28] but in agreement with results reported recently for rAPDK [20]. However, rCPDK did phosphorylate

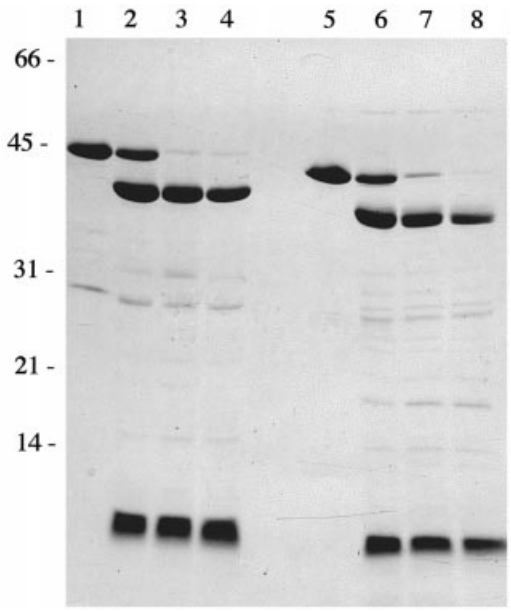

Figure 1 Limited tryptic digestion of rAPDK and rCPDK

PDKs were digested with trypsin for 5, 10 and 15 min, then separated by SDS/PAGE [15% (w/v) gel] and revealed with Coomassie Brilliant Blue R-250 as described in the Experimental section. Each lane contained 20 μg of protein. Lanes 1–4, rCPDK digested with trypsin for 0, 5, 10 and 15 min respectively; lanes 5–8, rAPDK digested with trypsin for 0, 5, 10 and 15 min respectively. The positions of molecular mass markers are indicated (in kDa) at the left.

Table 1 Effects of NADH and NADH plus acetyl-CoA on the activities of rAPDK and rCPDK

PDK assays were conducted as described in the Experimental section. Either rAPDK (0.05 μg) or rCPDK (0.05 μg) plus APDK-depleted PDC (15 μg) or intact PDC with endogenous kinase (20 μg) was preincubated for 1 min at 37 °C in 40 mM Tris/HCl (pH 7.4)/50 mM KCl/5 mM $MgCl_2$/5 mM dithiothreitol. The total NADH + NAD^+ concentration was constant at 800 μM. Activities are presented per mg of kinase protein for rAPDK and rCPDK, and per mg of total PDC protein for PDC. Results are means ± S.E.M. ($n = 4$).

	PDK activity					
	rAPDK		rCPDK		PDC	
Effectors	(nmol of ^{32}P/min per mg of PDK)	(fold increase)	(nmol of ^{32}P/min per mg of PDK)	(fold increase)	(nmol of ^{32}P/min per mg of PDC)	(fold increase)
NADH/NAD^+ (1:200)	320 ± 18	1.00	383 ± 22	1.00	1.12 ± 0.14	1.00
NADH/NAD^+ (3:1)	665 ± 30	2.08	578 ± 37	1.51	2.06 ± 0.16	1.84
NADH/NAD^+ (3:1) plus 50 μM acetyl CoA	924 ± 38	2.89	738 ± 20	1.93	3.00 ± 0.24	2.68

Table 2 Effect of limited tryptic digestion of rCPDK and rAPDK on PDK activity

Partial tryptic digestion of PDKs and PDK assays were conducted as described in the Experimental section. PDK (0.2 μg) activity was measured with native *A. suum* E1 (10 μg) or PDK-depleted PDC (20 μg). Numbers in parentheses are fold increase over PDK activity with E1 as a substrate. Results are means ± S.E.M. ($n = 3$).

			PDK activity (nmol of ^{32}P/min per mg of PDK)	
Digestion time (min)	PDK type	Substrate ...	E1	PDK-depleted PDC
0	rCPDK		94 ± 9	309 ± 28 (3.3)
	rAPDK		98 ± 13	365 ± 10 (3.7)
10	rCPDK		64 ± 6	80 ± 8 (1.2)
	rAPDK		61 ± 6	81 ± 6 (1.3)

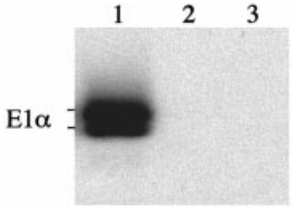

Figure 2 Autoradiography of native *A. suum* E1 and rCPDK incubated with [^{32}P]ATP

Purified rCPDK was incubated with *A. suum* E1 at 37 °C in 20 mM Tris/HCl (pH 7.4)/5 mM $MgCl_2$/50 mM KCl/5 mM dithiothreitol/Mg[γ-^{32}P]ATP^{2-} (2 mM $MgCl_2$/0.5 mM ATP; 150 c.p.m./pmol). After 1 h, samples (2 μg of E1 or 1 μg of rCPDK) were separated by SDS/PAGE [10% (w/v) gel] and processed for autoradiography as described in the Experimental section. Lane 1, E1 plus rCPDK; lane 2, rCPDK alone; lane 3, E1 alone. E1α: α-subunit of pyruvate dehydrogenase.

the native *A. suum* E1 and generated two phosphorylated E1 bands after autoradiography, as has been observed previously for phosphorylation of the native *A. suum* E1 [29,30]. Incubation of the native E1 alone in the absence of rCPDK resulted in no endogenous phosphorylation.

Limited proteolysis of rAPDK and rCPDK

Both nematode rPDKs were incubated with trypsin and the products of the digestion separated by SDS/PAGE (Figure 1). Limited tryptic digestion of both rAPDK and rCPDK yielded products of approx. 38 and 6–7 kDa for rCPDK and 37 and 5–6 kDa for rAPDK. Both tryptic fragments seemed to undergo additional proteolysis as the digestion continued. Chromatography of trAPDK and trCPDK on Superose 12 under non-denaturing conditions to isolate the major tryptic fragment yielded apparent molecular masses of approx. 72 and 68 kDa for trCPDK and trAPDK respectively, suggesting that the trPDKs retained their dimeric structure. N-terminal sequencing of the trPDK fragments indicated that the site of tryptic cleavage was at the C-terminus of the enzymes, because the N-termini of the larger tryptic fragments were intact. N-terminal sequencing of the truncated 6–7 kDa product indicated that the major site of tryptic cleavage occurred after Arg-313 (Figure 3). After proteolysis, both trPDKs still retained their ability to phosphorylate the *A. suum* E1 (at between 60–70 % of that of the corresponding rPDKs) (Table 2). However, this activity was not substantially stimulated by PDK-depleted *A. suum* PDC, suggesting that digestion might have interfered with the trPDKs' ability to bind to the E2 core (Table 2). The slight stimulation of PDK activity observed in the presence of PDK-depleted PDC by the trCPDK preparations is probably the result of residual undigested rCPDK (see Figure 1). Not surprisingly, trPDK activity was not stimulated significantly by NADH or acetyl-CoA (results not shown).

To examine the binding of rCPDK and trCPDK to the PDC directly, they were incubated with PDK-depleted PDC, which was then pelleted by centrifugation through a sucrose step-gradient. Because low amounts of PDK binding could not be detected directly after SDS/PAGE, rCPDK and trCPDK bound

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                                                  *
CPDK  274 VVKGQEDLSI KICDRGGGVS RTILERLYNY MYSTAPPPPR DGTQ APLAG YGYGLPLSRL YARYFLGDLF
APDK  273 ..R.A..... ..S..... ....D..FT. .......... .... P.... .......... .....H..MY
DPDK  281 IC..K..ICV ..S.Q...IP .SQTDQ.FK. ......Q.SK SDLHTV.... ......I... .....H..IV
HPDK1 305 .TL.N...TV .MS......P LRKID..F.. ........RV ETSRAV.... F.....I... ..Q..Q...K
HPDK3 274 .TL.K..... ..S.L....P LRKID..F.. ........SL EP.RA..... F.....I... .....Q...K
HPDK2 277 .AL.E..... .MS......P LRKI...FS. ......T.Q  P..GGT.... F.....I... ..K..Q...Q
HPDK4 280 ..L.K...T. ..S......P LR.ID..FS. T.....T.VM .NSRN..... F.....I... ..K..Q...N

CPDK      LVSMEGHGTD ACIYLKAVPV EASEVLPIYS TSSRRNLTMG PQVADWSHHV PGQGNRPAQS 402
APDK      ......Y... .M.F...I.. .......... .....Q...S ..A.....QL .NH...NL 399
DPDK      .L.C..F... .I.....LSD ..N.L...FN KT.SKFYRAT VPTG...NQV KYAKKKKTSA VSQ 413
HPDK1     .Y.L..Y... .V..I..LST DSI.R..V.N KAAWKHYNTN HEAD..CVPS REPKDMTTFR SA 436
HPDK3     .Y....V... .V.....LSS .SF.R..VFN K.AW.HYKTT .EAD...NPS SEPRDASKYK AKQ 406
HPDK2     .F....F... .V.....LST DSV.R..V.N K.AW.HYQTI QEAG..CVPS TEPK.TSTYR VT 407
HPDK4     .Y.LS.Y... .I.....LSS .SI.K..VFN K.AFKHYQ.S SEAD..CIPS REPK.LAKEV AM 411
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Figure 3 Alignment of the CPDK and APDK C-terminal regions with those of PDKs from other sources

C-terminal regions of PDKs from *C. elegans* (CPDK), *A. suum* (APDK), *Drosophila melanogaster* (DPDK) and *Homo sapiens* (HPDK1, HPDK2, HPDK3 and HPDK4) were aligned by using the GAP and PILEUP programs of the UWGCG, with final manual adjustments. Dots indicate identity with the CPDK sequence. The site of tryptic digestion is indicated with an asterisk.

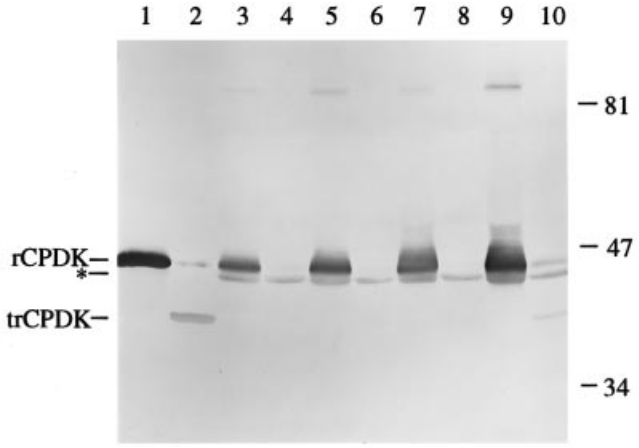

Figure 4 Binding of rCPDK and trCPDK to the PDK-depleted *A. suum* PDC

rCPDK and trCPDK prepared by limited tryptic digestion were incubated with PDK-depleted PDC, pelleted through a sucrose step-gradient, separated by SDS/PAGE [10% (w/v) gel], transferred to nitrocellulose and immunoblotted with affinity-purified polyclonal antiserum against purified rCPDK, as described in the Experimental section. Lane 1, rCPDK (0.5 μg); lane 2, trCPDK (0.5 μg); lanes 3, 5, 7 and 9 contained pelleted PDC (20 μg) previously incubated with 0.5, 1, 2 and 20 mol of rCPDK/mol of PDK-depleted PDC respectively; lanes 4, 6, 8 and 10 contained pelleted PDC (20 μg) previously incubated with 0.5, 1, 2 and 20 mol of trCPDK/mol of PDK-depleted PDC respectively. The asterisk indicates the position of p45 (E3-binding protein). The positions of molecular-mass markers are indicated (in kDa) at the right.

to the pelleted PDC were identified by immunoblotting with affinity-purified polyclonal antiserum against rCPDK. As illustrated in Figure 4, the addition of increasing amounts of rCPDK to the PDK-depleted PDC resulted in the appearance of increasing amounts of rCPDK in the pelleted PDC (Figure 4). No additional rCPDK binding was observed when rCPDK was incubated at ratios greater than 20 mol of rCPDK/mol of PDC. PDK activity in the pelleted PDC could not be accurately assayed at higher PDK concentrations, as the PDK reaction went rapidly to completion and true initial velocities could not be measured. In contrast, when trCPDK was incubated under identical conditions, little trCPDK sedimented with the PDC (Figure 4) and only a negligible amount of PDK activity was associated with the pelleted PDC (results not shown). At 20 mol of trCPDK/mol of PDC, very limited amounts of trPDK were observed with the pelleted PDC, perhaps associated with an undigested subunit of the rCPDK homodimer, because some undigested rCPDK was present in these incubations (Figure 4).

The anti-rCPDK antiserum used in these studies strongly recognized the 6–7 kDa tryptic fragment and more weakly the 38 kDa tryptic fragment. In addition, this antiserum also weakly recognized the 45 kDa component of *A. suum* PDC (p45), as has been observed previously for antisera prepared against rAPDK [20]. The reasons for this cross-reactivity are unclear. When tryptic digests of rCPDK that contained both the 38 and 6–7 kDa fragments were incubated with the PDK-depleted PDC, no evidence for the binding of the 6–7 kDa fragment was observed (results not shown). Excess bovine trypsin inhibitor was added to all samples and prevented any potential proteolysis of the trypsin-sensitive E2 and E1 subunits of the PDC during separation and assay, as indicated by the lack of degradation of these subunits on Coomassie Blue-stained SDS/polyacrylamide gels of the pelleted PDCs.

Mutagenesis to examine the role of the C-terminus in the binding of rCPDK to the PDC

To examine the potential roles of the N- and C-termini of the nematode rPDKs in catalysis and binding, truncated forms of rCPDK were generated by mutagenesis and then purified by chromatography on Ni-NTA-agarose. When the N-terminal 50 residues of rCPDK were deleted, the resulting truncated rCPDK was catalytically inactive. In contrast, deletion of the C-terminal 68 residues yielded a truncated form of rCPDK containing residues 1–334 ($rCPDK_{1–334}$) with substantial PDK activity (approx. 80 nmol/min per mg of protein with E1). More importantly, PDK activity was not increased when PDK-depleted PDC was used as a substrate. These results confirm the results obtained by tryptic digestion, suggesting that the C-terminus is not involved directly in catalysis but instead in the binding of the PDK to the E2 core. $rCPDK_{1–334}$ was much less soluble than rCPDK and recoveries after purification were less than 100 μg of protein/l of bacterial culture. The co-expression of GroEL and GroES did not increase the amount of protein in the soluble fraction. Mutant rCPDKs truncated at the C-terminus after Tyr-301, Pro-312 and Val-362 were insoluble and were not studied further.

Native and recombinant mammalian PDKs exist as dimers and seem to move between lipoyl domains without dissociation, as recently described by a 'hand-over-hand' model [15]. This model predicts that each PDK homodimer should have two lipoyl-binding domains and suggests that an individual 'binding site' might be sufficient for association with the PDC. To examine

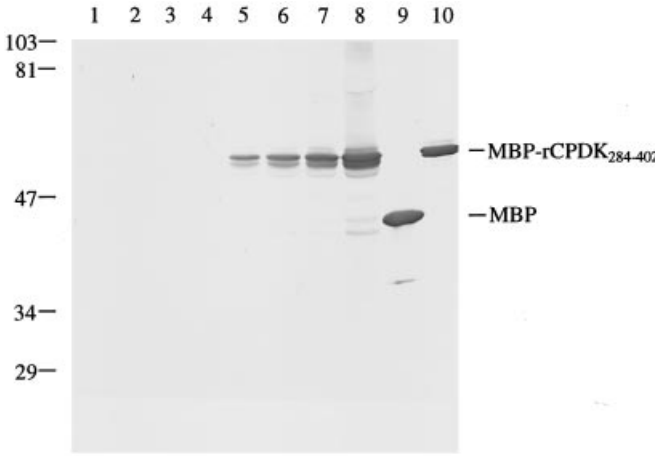

Figure 5 Binding of MBP and MBP–rCPDK$_{284–402}$ to PDK-depleted PDC

MBP and MBP–rCPDK$_{284–402}$ were incubated separately with PDK-depleted PDC, pelleted through a sucrose step-gradient, separated by SDS/PAGE [10% (w/v) gel], transferred to nitrocellulose and immunoblotted with monoclonal antibodies against MBP, as described in the Experimental section. Lanes 1–4 contained pelleted PDC (20 μg) incubated with 1, 2, 5 and 20 mol of MBP/mol of PDK-depleted PDC respectively; lanes 5–8 contained pelleted PDC (20 μg) incubated with 1, 2, 5 and 20 mol of MBP–rCPDK$_{284–402}$/mol of PDK-depleted PDC respectively. MBP (0.5 μg, lane 9) and MBP–rCPDK$_{284–402}$ (0.5 μg, lane 10) are included as controls. The positions of molecular mass markers are indicated (in kDa) at the left.

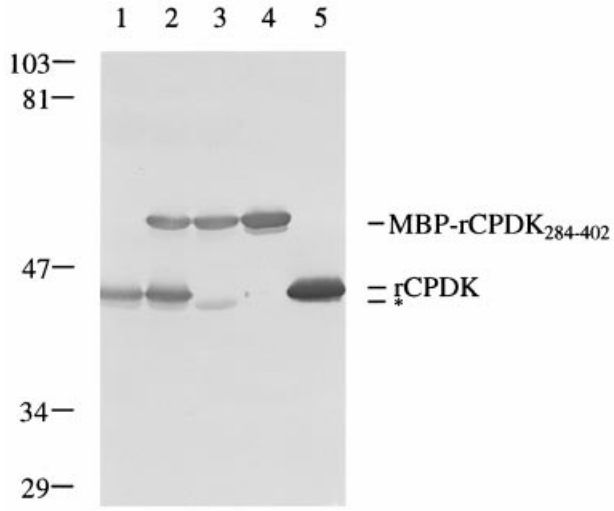

Figure 6 Binding of rCPDK and MBP–rCPDK$_{284–402}$ to PDK-depleted PDC

MBP–rCPDK$_{284–402}$ and/or rCPDK were incubated with PDK-depleted PDC, pelleted through a sucrose step-gradient, separated by SDS/PAGE [10% (w/v) gel], transferred to nitrocellulose and immunoblotted with affinity-purified polyclonal antibodies against the purified rCPDK, as described in the Experimental section. PDK-depleted PDC (20 μg) was pelleted after incubation with 5 mol of rCPDK/mol of PDK-depleted PDC (lane 1), with 5 mol of rCPDK/mol of PDK-depleted PDC plus 5 mol of MBP–rCPDK$_{284–402}$/mol of PDK-depleted PDC (lane 2) and with 5 mol of MBP–rCPDK$_{284–402}$/mol of PDK-depleted PDC (lane 3). MBP–rCPDK$_{284–402}$ (0.5 μg, lane 4) and rCPDK (0.5 μg, lane 5) are included as controls. The asterisk indicates the position of p45 (E3 binding protein). The positions of molecular-mass markers are indicated (in kDa) at the left.

this possibility more directly, the putative rCPDK E2-binding domain, identified by limited proteolysis and mutagenesis, was expressed at the C-terminus of MBP (MBP–rCPDK$_{284–402}$) and purified by affinity chromatography; the purified MBP–rCPDK$_{284–402}$ was incubated with PDK-depleted PDC. On the basis of immunoblotting analysis as described for rCPDK, MBP–rCPDK$_{284–402}$ seemed to bind to PDK-depleted PDC (Figure 5). As observed for the binding of rCPDK to the PDC, no additional MBP–rCPDK$_{284–402}$ binding was observed at ratios greater than 20 mol of MBP–rCPDK$_{284–402}$/mol of PDC. In contrast, MBP incubated under identical conditions did not bind to the PDC (Figure 5), nor did MBP–rCPDK$_{284–402}$ sediment when incubated alone, suggesting that this fusion construct did not aggregate during incubation (results not shown).

These observations might suggest that the C-terminus of rCPDK (residues 284–402) alone might be sufficient for binding to the PDC. However, when rCPDK and MBP–rCPDK$_{284–402}$ were incubated together at 5 mol/mol of PDC, neither interfered with the binding of the other, as judged by immunoblotting of the pelleted PDC (Figure 6). In fact, preincubation of the PDK-depleted PDC with excess rCPDK (40 mol/mol of PDC) before the addition of MBP–rCPDK$_{284–402}$ (5 mol/mol of PDC) or incubation of excess MBP–rCPDK$_{284–402}$ before the addition of rCPDK also had no significant effects on the binding of either MBP–rCPDK$_{284–402}$ or rCPDK (results not shown). A likely explanation for this observation is that MBP–rCPDK$_{284–402}$ binding is non-specific, i.e. that MBP–rCPDK$_{284–402}$ and rCPDK might be binding to a different sites on the PDC and that the C-terminus alone might not be sufficient for specific binding. However, the binding of both proteins seems to saturate at similar stoichiometries and MBP–rCPDK$_{284–402}$ does not dissociate when the complex is incubated in 2 M NaCl as has been observed previously for the tight binding of the native PDKs to the E2 core (results not shown). The MBP–rCPDK$_{284–402}$ fusion protein with only a single putative binding region might have access to PDK-binding sites that are not available to rCPDK.

DISCUSSION

PDKs from the parasitic nematode *A. suum* and the free-living nematode *C. elegans* have been functionally expressed and purified and their regulatory properties have been compared when bound to a PDK-depleted PDC isolated from body wall muscle of adult *A. suum*. The physiological environments of these two PDKs are quite different. *C. elegans* exhibits a classical aerobic metabolism, whereas *A. suum* is anaerobic and uses unsaturated organic acids as terminal electron-acceptors [31]. Anaerobic mitochondrial metabolism in *A. suum* is characterized by elevated ratios of NADH to NAD^+ and of acetyl-CoA to CoA, and by a reversal of β-oxidation that results in the formation of novel branched-chain fatty acids as end products of carbohydrate metabolism [32,33]. Surprisingly, the APDK was still quite sensitive to stimulation by NADH and acetyl-CoA despite the fact that these ratios are markedly elevated during anaerobiosis. However, APDK did require higher NADH/NAD^+ ratios for half-maximal stimulation than rCPDK or those reported for the mammalian PDKs (0.03 and 0.05 for pig liver and bovine kidney PDK respectively, as compared with 0.21 for rAPDK in the present study) [34]. This observation is probably not surprising, given the markedly different intramitochondrial redox environments in which the PDKs are found. Stimulation by NADH and acetyl-CoA is dependent on the catalytic formation of acetyl dihydrolipoate by the PDC and is mediated by the E3-catalysed reduction and E2-catalysed acetylation of the lipoyl prosthetic group of E2 [15]. In mammalian PDCs, E3BP also contains an N-terminal lipoyl domain, although its role in the binding and regulation of the PDKs is unclear [35]. Interestingly, the *A. suum* E3BP lacks a terminal lipoyl domain, suggesting that an E3BP lipoyl domain is not necessary for the regulation of PDK activity by NADH or acetyl-CoA, at least in

the *A. suum* PDC [21]. Theoretically, the sensitivity of the PDK to NADH could be an innate property of the PDK, or a function of E2 or E3. In the present study, both nematode PDKs were assayed bound to the *A. suum* PDK-depleted PDC, meaning that both PDKs utilized the same E2 and E3. However, recent studies with the four mammalian PDK isoenzymes have suggested that the PDKs themselves also exhibit marked differences in specific activity and in sensitivities to stimulation by NADH and acetyl-CoA [9]. In that study, the rat PDK1, PDK2 and PDK4 and the human PDK3 were reconstituted with PDK-depleted bovine kidney PDC. The possible role of species differences in these hybrid complexes was not addressed. The use of heterologous reconstitution systems has recently been questioned [36]. For example, the yeast E3 binds to the bovine kidney E2 core, but does not seem to be oriented properly for catalysis; it has been suggested that the inability of previous studies to demonstrate PDC activity after selective proteolysis of E3BP might have been the result of the use of heterologous pig heart E3 in the reconstitution studies [37].

As described above, the stimulation of PDK activity by either NADH or acetyl-CoA first requires the binding of the PDK to the E2 core. The mammalian E2 contains two distinct lipoyl domains (L1 and L2) located at the N-terminus [38,39]. It has been clearly demonstrated that the mammalian PDK binds to the internal lipoyl domain (L2), perhaps through a highly charged region at the C-terminus, and PDK binding requires the presence of the lipoyl prosthetic group [4,12,40]. In contrast, nothing is known about the structure or region of PDK responsible for interacting with E2. In the present study we have demonstrated that limited tryptic digestion of both the nematode rPDKs yields stable 37–38 kDa fragments that retain PDK activity with E1 as substrate but are not stimulated by PDK-depleted PDC and do not associate with the E2 core in sedimentation experiments. The failure of tryptic digestion to abolish rCPDK activity might explain why tryptic digestion of the 45 kDa 'regulatory' subunit of the native bovine kidney PDK had no effect on PDK activity, and suggests the possibility that both the 45 and 48 kDa subunits of the native bovine kidney PDK are involved in catalysis [41].

The putative E2-binding domain of the PDK is located at the C-terminus and, in the nematode PDKs at least, is separated from the remainder of the molecule by a proline-rich region (Ser-Thr-Ala-Pro-Pro-Pro-Pro; Figure 3). In addition, because the nematode trPDKs still seem to be dimers, as judged by chromatography on Superose 12, this C-terminal domain does not seem to be involved in subunit association. A number of conserved domains were identified when the sequences of PDKs from different sources were compared [7]. Interestingly, sequence conservation is present in the C-terminal binding region among all these PDKs (Figure 3). However, similarity in this region is not apparent in putative PDK sequences from the yeast *Saccharomyces cerevisiae* and the African trypanosome *Trypanosoma brucei* [42]. In agreement with this observation, there is also no evidence that PDCs from *S. cerevisiae* or a closely related trypanosomatid, *Crithidia fasiculata*, contain PDK activity [26]. In addition, the putative trypanosome PDK lacks a conserved glycine-rich loop in the proposed catalytic domain [42]. These studies are continuing to characterize more fully the E2-binding domain of the nematode PDKs by site-directed mutagenesis.

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