Interaction between the individual isoenzymes of pyruvate dehydrogenase kinase and the inner lipoyl-bearing domain of transacetylase component of pyruvate dehydrogenase complex

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Protein–protein interactions play an important role in the regulation of enzymic activity of pyruvate dehydrogenase kinase (PDK). It is generally believed that the binding of PDK to the inner lipoyl-bearing domain L2 of the transacetylase component E2 of pyruvate dehydrogenase complex largely determines the level of kinase activity. In the present study, we characterized the interaction between the individual isoenzymes of PDK (PDK1–PDK4) and monomeric L2 domain of human E2, as well as the effect of this interaction on kinase activity. It was found that PDK isoenzymes are markedly different with respect to their affinities for L2. PDK3 demonstrated a very tight binding, which persisted during isolation of PDK3–L2 complexes using size-exclusion chromatography. Binding of PDK1 and PDK2 was readily reversible with the apparent dissociation constant of approx. 10 μM for both isoenzymes. PDK4 had a greatly reduced capacity for L2 binding (relative order PDK3 > PDK1 = PDK2 > PDK4). Monomeric L2 domain alone had very little effect on the activities of either PDK1 or PDK2. In contrast, L2 caused a 3-fold increase in PDK3 activity and approx. 37% increase in PDK4 activity. These results strongly suggest that the interactions between the individual isoenzymes of PDK and L2 domain are isoenzyme-specific and might be among the major factors that determine the level of kinase activity of particular isoenzyme towards the pyruvate dehydrogenase complex.

Key words: carbohydrate metabolism, mitochondria, oxidative decarboxylation of pyruvate, protein phosphorylation, protein–protein interactions.

INTRODUCTION

Oxidative decarboxylation of pyruvate, pyruvate + NAD^+ + CoA → CO_2 + NADH + acetyl-CoA serves as an important link connecting two major catabolic pathways, namely glycolysis and the tricarboxylic acid cycle. The overall reaction is catalysed by the pyruvate dehydrogenase complex (PDC). PDC is a multienzyme complex, which consists of three catalytic components, namely the pyruvate dehydrogenase component E1 (tetramer of two α and two β subunits), the dihydrolipoyl acetyltransferase component E2 [consists of 60 subunits of transacetylase and 12 subunits of E3-binding protein component (E3BP)] and the dihydrolipoamide dehydrogenase component E3 (dimer of two α subunits) [1]. The complex is built around the core of transacetylase component assembled as a pentagonal dodecahedron [2].

It is generally believed that the enzymic activity of PDC determines the rate of aerobic oxidation of carbohydrates [3]. In mammals, this flux-generating function of PDC is regulated by a sophisticated phosphorylation/dephosphorylation cycle [1]. Inactivation of PDC occurs as a result of phosphorylation of three seryl residues (sites 1–3) located on the α chain of the E1 component, with site 1 being the primary inactivating site [4]. Phosphorylation is catalysed by a dedicated pyruvate dehydrogenase kinase (PDK) [5]. Phospho-PDC is re-activated through dephosphorylation, catalysed by the dedicated pyruvate dehydrogenase phosphatase (PDP) [6]. The activities of both kinase and phosphatase components are tightly regulated [1]. PDK activity increases in the presence of elevated concentrations of NADH and acetyl-CoA, whereas elevated concentrations of NAD^+, CoA [7] and pyruvate inhibit kinase activity [8]. PDP, in contrast, is inhibited by elevated concentrations of NADH and is activated in the presence of calcium ions [9,10]. The relative activities of PDK and PDP in mitochondria determine the proportion of PDC in its active dephosphorylated state (activity state). Depending upon the existing metabolic requirements, the activity state of PDC can change from approx. 70% (30% of phosphorylated, inactive PDC) to as low as 1–2% (98–99% of phosphorylated, inactive PDC) [11].

In contrast to PDP, which associates with PDC only transiently [12], PDK is an integral part of the complex [13]. The docking sites for kinase molecule are provided by the inner lipoyl-bearing domains L2 of the transacetylase component E2 [14–16]. The association with E2 is critically important for the kinase function. Studies on wild-type PDK of unspecified isoenzymic composition have established that the addition of E2 causes a several-fold increase in PDK activity, and therefore the presence of E2 largely determines the rates of phosphorylation and inactivation of pyruvate dehydrogenase [6]. The molecular mechanism responsible for the kinase activation caused by addition of E2 has not been elucidated. Indirect evidence suggests that this mechanism has a rather complex nature. It might include conformational changes in the structures of kinase and its substrate E1, as well as the effects of co-localization and mutual orientation caused by transacetylase binding [17]. Another potentially important consequence of the interaction between kinase and E2 is that it provides the means for allosteric control of kinase activity by NADH/NAD^+ and acetyl-CoA/CoA [18]. It is generally believed that the effects of NAD^+, NADH, CoA and acetyl-CoA on PDK activity are not direct. They are transmitted to the kinase molecule

Abbreviations used: DTT, dithiothreitol; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase.

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by the lipoate prosthetic groups attached to the outer (L1) and inner (L2) lipoyl-bearing domains of the E2 component [7]. The oxidized lipoates (reflective of the high concentrations of NAD^+ and CoA) inhibit phosphorylation of E1 by PDK, whereas the reduced and acetylated lipoates (reflective of the high concentrations of NADH and acetyl-CoA) facilitate phosphorylation [7]. The kinase acquires the ability to sense the changing states of lipoates, presumably to the docking to L2 domains [14,16]. This makes L2 the domain of central importance in the regulation of kinase activity. At present, four isoenzymes of PDK have been identified in mammalian tissues [19,20]. The interactions between the individual isoenzymes of PDK and L2 domain are poorly characterized. For example, Mann et al. reported that neither PDK1 nor PDK2 could bind to the L2 domain [21]. On the other hand, Baker et al. [22] have found that the monomeric L2 domain can directly activate PDK3 at low micromolar concentrations. The slight activation of PDK2 was observed by these authors with a dimeric construct of the L2 domain, suggesting that both isoenzymes can interact with L2. It should be pointed out that all the three isoenzymes tested so far are likely to exist in a PDC-bound form, as evidenced by their co-purification with rat heart [23] and bovine kidney complexes [22]. This somewhat uncertain outcome prompted us to investigate the interactions between the individual isoenzymes of mammalian PDK and the L2 domain.

EXPERIMENTAL

Bacterial strains and plasmids

Plasmids for the expression of His_6-tagged PDK1–PDK4, as well as for the expression of transacetylase component and wild-type E1 component were described previously (vectors pH6K1, pH6K2, pH6K3, pH6K4, pE2/E3BP and pWTE1 respectively) [24,25]. The plasmid for the expression of His_6-tagged L2 (H6L2) was constructed as follows: cDNA encoding L2 (bases 544–808 of cDNA coding for human E2 component [25]) was amplified using *Pfu* DNA polymerase. Primers for amplification (upstream primer: CCTCATATGTCATATCCCCCTCACATGCAGGTA, bases 544–568; downstream primer: AGCCTCGAG TTATATATCTGCCTCTTTTTCTACAAT, bases 781–808) contained unique *Nde*I and *Xho*I restriction sites (underlined). The 281-bp-long cDNA was subcloned into pUC19 vector cut with *Sma*I and dephosphorylated with calf intestine phosphatase. Fidelity of the cDNA was confirmed by sequencing [26]. *Nde*I/*Xho*I fragment of L2 cDNA was cut from pUC19 and ligated between *Nde*I and *Xho*I sites of pET-28a expression vector (Calbiochem-Novabiochem, San Diego, CA, U.S.A.) producing an amino-terminal fusion with His_6-tag encoded by the vector pH6L2. To obtain the untagged L2, its cDNA was subcloned between *Nde*I and *Xho*I sites of pET-23a expression vector pWTL2 obtained from Calbiochem-Novabiochem. To construct the expression vectors for wild-type isoenzymes of PDK, unique restriction sites were introduced in the respective cDNAs [24], which are as follows: for PDK1 cDNA, *Nde*I (base 73) and *Xho*I (base 1306) sites; for PDK2 cDNA, *Nde*I (base 19) and *Xho*I (base 1225) sites; for PDK3 cDNA, *Nhe*I (base 41) and *Xho*I (base 1271) sites; and for PDK4 cDNA, *Nco*I (base 130) and *Hin*dIII (base 1370) sites. The sequences of oligonucleotide primers are as follows: for PDK1 upstream primer, AAACATATGGCCTCGGACTCGGCCTCGGGC; for PDK1 downstream primer, AAAAAGCTTTTACGAGCTTCGGAACGTCGT; for PDK2 upstream primer, AAACATATGAAGAATGCGTCCCTGGCA; for PDK2 downstream primer, TTTCTCGAGCTAGCTGACCCGATACGTCGATGT; for PDK3 upstream primer, TGGGCTAGCAAGCAGCCGGTGCCCAAGCAG; for PDK3 downstream primer, AATCTCGAGCTTTGTAATGGAAATCAAGGTGG; for PDK4 upstream primer, AAACCATGGCCAGCTCGCTGGGCAACG; for PDK4 downstream primer, ATAAAGCTTCGTAAAAGTGCCAGGCGAGAT (the corresponding restriction sites are underlined). Resulting cDNAs were subcloned into pUC19 vector (cut with *Sma*I and dephosphorylated with calf intestinal alkaline phosphatase) and sequenced [26]. After digestion with appropriate restriction enzymes, the cDNAs were subcloned into the following expression vectors: PDK1 into pET-30a (vector pWTK1); PDK2 into pET-30a (vector pWTK2); PDK3 into pET-21a (vector pWTK3); and PDK4 into pET-28a (vector pWTK4). Finally, the plasmid for expression of His_6-tagged E1 component (vector pH6E1) was constructed essentially as described for pWTE1 vector [27], except that cDNA for E1α flanked by unique *Nde*I and *Sal*I restriction sites was subcloned into pET-28a vector instead of pET-21a (vector pH6E1A) to produce the amino-terminal fusion with His_6-tag encoded by the vector. The expression cassette for E1β subunit carrying T_7 promoter, ribosome-binding site and T_7 terminator was cut from pWTE1B vector with *Dra*III and *Sph*I restrictases. The DNA (approx. 1.7 kb) was blunt-ended using T_4 polymerase and subcloned into pH6E1A vector cut with *Dra*III and blunt-ended with T_4 polymerase. Plasmids carrying both cDNAs were identified by restriction analysis. All enzymes used for the construction of the respective expression vectors were purchased from New England Biolabs (Beverly, MA, U.S.A.). Oligonucleotides were obtained from Life Technologies (Rockville, MD, U.S.A.). Bacterial expression vectors were from Calbiochem-Novabiochem.

Growth of cells and protein purification

General conditions for the expression of His_6-tagged PDK1–PDK4, as well as E1 and transacetylase were described previously [24,25]. Wild-type kinases, His_6-tagged E1, untagged L2 and His_6-tagged L2 were expressed under similar conditions. Briefly, appropriate plasmids were transformed into BL21 (DE3) cells along with the plasmid carrying the genes encoding the molecular chaperones GroEL and GroES under the control of isopropyl β-D-thiogalactoside-inducible promoter (the respective plasmid was obtained as a gift from Dr Anthony Gatenby at DuPont Central Research and Development, Wilmington, DE, U.S.A.). Double transformants were selected on M9ZB agar supplemented with appropriate antibiotics. Several individual colonies from each transformation were tested for their ability to produce significant amounts of soluble recombinant protein. Clones expressing the highest amount of the soluble enzyme were used for further analysis. For large-scale protein expression, respective clones were inoculated into 1 litre of M9ZB medium supplemented with the appropriate antibiotics. Cultures were grown at 37 °C with continuous shaking at 200 rev./min until A_{600} reached approx. 0.6. Cultures were cooled on ice and transferred into the shaker-incubator set at 200 rev./min and temperature of 18 °C. Expression was induced with isopropyl β-D-thiogalactoside at a final concentration of 0.4 mM for 18 h. Cells were harvested by centrifugation at 5000 rev./min (JA-10 rotor) for 30 min at 4 °C. For expression of wild-type E1, His_6-tagged E1, L2 and His_6-tagged L2, cultures were supplemented with thiamine (50 μg/ml) and lipoic acid (0.2 mM) respectively before induction. When unlipoylated L2 was produced, the lipoic acid was omitted from the growth media.

Purification of His_6-tagged PDK1, PDK2, PDK3 and PDK4, as well as of the E1-transacetylase complex are described else-

where [24,25]. Purification of transacetylase alone was performed essentially as described for E1-transacetylase complex using polyethylene glycol 8000 precipitation, gel-filtration on Sepharose 4B and high-speed centrifugation [25]. Purification of His_6-tagged E1 and L2 was achieved by metal affinity chromatography on 1 ml of TALON™ resin (Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.) following the manufacturer's instructions. L2 and E1 were eluted from the affinity matrix using buffers containing 50 mM and 100 mM imidazole respectively.

Chromatography

To determine the molecular mass and stoichiometry of PDK–L2 complexes, the respective preparations were subjected to gel filtration on a Sephadex G-150 (superfine) column (bed volume of 1.6×35 cm) equilibrated in 10 mM sodium phosphate (pH 7.4), 138 mM NaCl, 2.7 mM KCl + 5 mM D,L-dithiothreitol (DTT). Samples (250 μl) were injected on the column at a flow rate of 0.4 ml/min at room temperature. Elution was monitored spectrophotometrically at 280 nm. Fractions (0.5 ml) were collected and analysed using SDS/PAGE followed by Coomassie Blue R-250 staining. Protein content of each fraction was determined by scanning densitometry of Coomassie Blue-stained gels. When the stoichiometry of Sephadex G-150-purified PDK3-L2 was determined, the complex was separated on SDS/PAGE along with BSA used as a protein standard. BSA was loaded at 0.07, 0.15, 0.3, 0.6, 1.2 and 2.4 μg/lane. Based on the results of scanning densitometry a calibration curve was constructed, which was used for the determination of the absolute amounts of PDK3 and L2 in the complex. For molecular-mass determination, a Sephadex G-150 column was calibrated with molecular-mass standards (catalase, 232 kDa; aldolase, 158 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa; ribonuclease A, 13.7 kDa).

The 'equilibrium chromatography' method of Hummel and Dreyer [28] was used to characterize the protein–protein interactions of PDK1, PDK2 and L2. A Superose 12-prepacked HR 10/30 gel filtration column (included volume of 18 ml) connected to an FPLC system (Pharmacia, Piscataway, NJ, U.S.A.) was equilibrated with purified L2. A 100 μl injection of PDK1 or PDK2 plus concentration of L2 equal to or greater than the column concentration was pumped to the column at the rate of 0.2 ml/min. Absorbance of the effluent was monitored at 280 nm with a Pharmacia UV-MII monitor. Relative areas of trough and peak recorded with Pharmacia REC112 recorder were quantified using Un-Scan-It software (Silk Scientific, Inc.). Binding experiments were conducted at room temperature. The Hummel–Dreyer binding buffer contained 20 mM Tris/HCl (pH 7.8), 5 mM $MgCl_2$, 50 mM KCl and 5 mM DTT.

Phosphorylation assay

General conditions for the phosphorylation assay based on the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into the E1α subunit were described previously [24]. Briefly, components of the reaction cocktail were mixed in a final volume of 45 μl and allowed to equilibrate for 30 min on ice. By the end of the incubation, mixtures were equilibrated at 37 °C for 60 s. Phosphorylation reactions were initiated by the addition of 0.4 mM [γ-^{32}P]ATP (specific radioactivity of 100–200 c.p.m./pmol). ATP was introduced into one-tenth of the total reaction volume. The final concentrations of the components in phosphorylation cocktail were as follows: 20 mM Tris/HCl (pH 7.8), 5 mM $MgCl_2$, 50 mM KCl, 1 mM 2-mercaptoethanol, 5 μM E1, 20 nM of appropriate kinase and 20 nM transacetylase or 80 μM L2. After 30 s incubation, 40 μl aliquots were quenched on Whatman 3MM filters pre-soaked in a solution of 20 % (w/v) trichloroacetic acid, 50 mM sodium pyrophosphate and 50 mM ATP. After extensive washing, the protein-bound radioactivity was determined by liquid-scintillation counting. A negative control (−PDK) was used to determine non-specific incorporation. All assays were conducted in triplicates.

Other procedures

The activity of PDC was determined as described previously [25]. SDS/PAGE was performed according to Laemmli [29]. Protein concentrations were determined according to Lowry et al. [30] with BSA as the standard. Western-blot analysis was performed as described elsewhere [31]. Rabbit antibodies raised against recombinant PDK4 or PDC were used at a final dilution of 1:1000. Immunoreactive bands were visualized using the ECL® immunodetection procedure (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) following the manufacturer's instructions. Secondary goat anti-rabbit antibodies conjugated with horseradish peroxidase were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A). They were used at a working dilution of 1:3000.

RESULTS

Isolation of individual isoenzymes of PDK on His_6-tagged L2 construct

As discussed in the Introduction section, the interactions between the individual isoenzymes of PDK and L2 domain remain poorly characterized. In order to establish that a particular isoenzyme of PDK is capable of binding to L2, we attempted to isolate the respective PDK–L2 complexes. L2 domain is a compact, somewhat flattened β barrel flanked at the amino- and carboxy-terminal ends by long poorly ordered linkers [32]. To use L2 domain of human PDC as a 'bait' for affinity isolation of PDKs, a stretch of six consecutive histidine residues (His_6-tag) was constructed at the amino-terminal end of the linker. In this construct, the affinity tag is separated from the L2 domain by an approx. 10-amino-acid-long spacer [32]. Plasmids for expression of mammalian PDKs were constructed to direct the synthesis of mature, untagged polypeptides of the respective kinases. L2 and kinase isoenzymes were expressed in *Escherichia coli* cells under the control of a T_7 promoter (see the Experimental section). The recombinant proteins were extracted from harvested cells and their amounts in the soluble fraction were estimated by Western-blot analysis (results not shown). Based on the results of this analysis, the volumes of the extracts were adjusted to ensure that each binding mixture received the appropriate kinase and L2 domain at comparable concentrations. After 30 min of reconstitution on ice, PDK–L2 mixtures were loaded on to 1 ml TALON affinity columns equilibrated in TN buffer [50 mM Tris/HCl (pH 8.0), 100 mM NaCl]. Columns were washed extensively with a TN buffer containing an increasing concentration of imidazole (0–10 mM). Proteins bound specifically were eluted with TN buffer containing 100 mM imidazole. The protein composition of the samples was analysed by SDS/PAGE (Figure 1A). The results of this analysis clearly demonstrated that three out of four isoenzymes of PDK (PDK1, PDK2 and PDK3 respectively) could be readily isolated on the His_6-tagged construct of the L2 domain. This suggests that these isoenzymes have a capacity to bind to an L2 domain. The binding is sufficiently strong to allow for affinity purification. In marked contrast, the amount of PDK4 recovered with L2 was substantially lower (Figure 1A). This observation indicates that PDK4 has a lower affinity for L2. To explore this hypothesis further, we attempted to isolate the un-

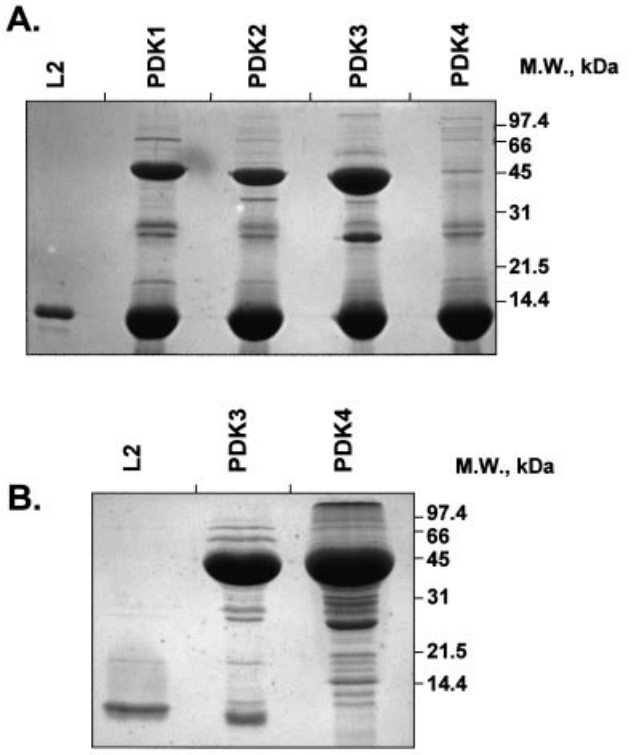

Figure 1 SDS/PAGE analysis of affinity-purified PDKs

(**A**) Affinity isolation of individual isoenzymes of PDK on His_6-tagged L2 domain construct. (**B**) Affinity isolation of untagged L2 domain on His_6-tagged PDK3 and PDK4 constructs. PDKs were complexed with L2; complexes were isolated using metal-affinity chromatography on TALON™ resin. Aliquots of kinase containing fractions were supplemented with 5 × SDS/PAGE loading buffer. Samples (5 μl) were separated in 15% polyacrylamide gel. The gel was stained with Coomassie Blue R-250.

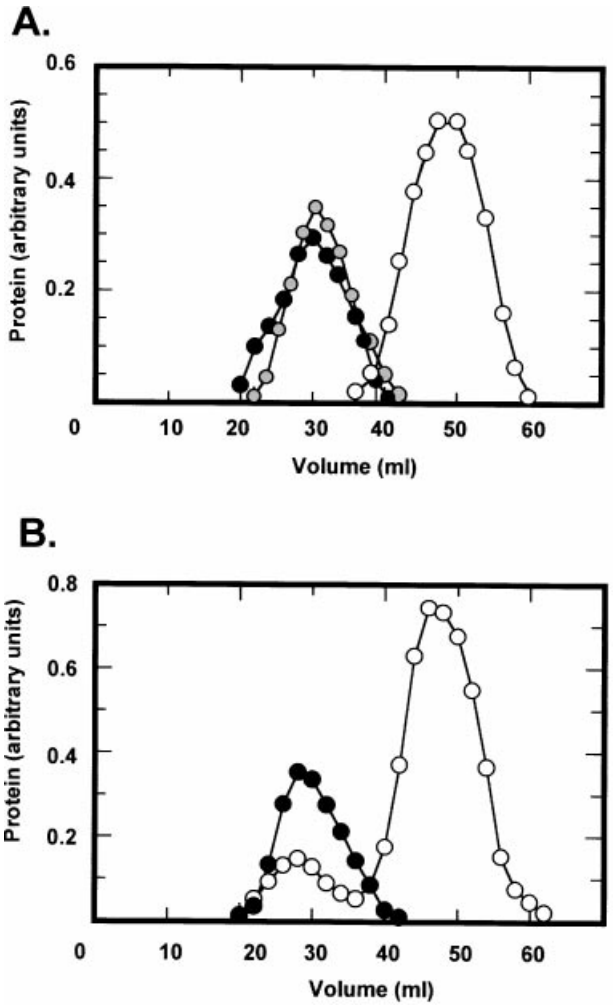

Figure 2 Chromatography of PDK–L2 complexes on Sephadex G-150 column

(**A**) Elution profile of PDK1–L2 and PDK2–L2 complexes. PDK1–L2 and PDK2–L2 complexes (250 μl) in PBS plus 5 mM DTT were chromatographed on Sephadex G-150 (superfine) column (1.6 × 35 cm) equilibrated in PBS supplemented with 5 mM DTT. Fractions (0.5 ml) were collected. Protein content of the fractions was analysed after separation of 20 μl aliquots of each fraction on 15% polyacrylamide gels. Gels were stained with Coomassie Blue R-250. Proteins were quantified using scanning densitometry. Closed circles, position of PDK1; closed circles, position of PDK2; open circles, position of L2 domain. (**B**) Elution profile of PDK3–L2 complex separated on Sephadex G-150 column. Closed circles, position of PDK3; open circles, position of L2.

tagged L2 domain on His_6-tagged PDK4 construct. As a control, similar experiments were performed with His_6-tagged PDK3. SDS/PAGE analysis of the respective samples demonstrated the presence of the L2 domain in PDK3-containing fractions (Figure 1B). The presence of L2 in PDK4-containing fractions could be detected only by Western-blot analysis (results not shown), providing further evidence that PDK4 has a reduced capacity for L2 binding. Interestingly, analysis of five independent preparations of kinases performed during the course of the present study consistently showed that the amount of PDK3 isolated on the L2 construct was approx. 1.5–2-fold greater than that of PDK1 or PDK2. This indicates that PDK3 binds to the L2 domain more strongly than any other isoenzyme. Together, these results are consistent with the hypothesis that the affinity of individual isoenzymes of PDK for the L2 domain decreases in the order PDK3 > PDK1 = PDK2 > PDK4.

Gel filtration on Sephadex G-150 column

To explore further the strength of interactions between the individual isoenzymes of PDK and L2 domain, the respective complexes obtained by affinity chromatography were subjected to gel filtration on Sephadex G-150 column as described in the Experimental section. Proteins eluted from the column were collected in fractions of 0.5 ml. The protein composition of individual fractions was analysed by SDS/PAGE followed by Coomassie Blue staining. The relative intensities of protein bands were determined by scanning densitometry. As shown in Figure 2(A), chromatography of PDK1–L2 or PDK2–L2 complexes resulted in a complete separation of the respective kinases and L2 domain. In fact, we were unable to detect L2 in kinase-containing fractions even on the gels stained with silver (results not shown). In marked contrast, the L2 domain was readily detectable in PDK3-containing fractions (Figure 2B). These observations are consistent with the hypothesis that binding of L2 to either PDK1 or PDK2 is readily reversible and that the respective complexes dissociate during separation, whereas the strength of the interaction between PDK3 and L2 is substantially greater and the respective complex can be isolated by gel filtration. This hypothesis is also corroborated by the fact that the molecular mass of PDK1 and PDK2 eluted from Sephadex G-150 column was approx. 90 kDa, corresponding

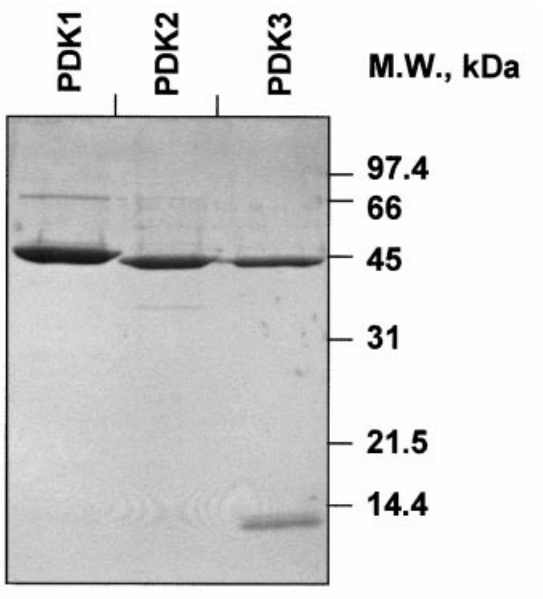

Figure 3 Stoichiometry of PDK3–L2 complexes isolated by gel filtration on Sephadex G-150 column

Line 1, SDS/PAGE analysis of the fraction number 28 corresponding to the maximum of PDK3-containing peak; lines 2 and 3, fractions corresponding to the maxima of PDK1 (fraction 30) and PDK2 (fraction 30)-containing peaks. Aliquots (20 μl) of the indicated fractions were separated on 15% polyacrylamide gel. The gel was stained with Coomassie Blue R-250.

to the molecular mass of free PDK dimer. The molecular mass of PDK3 under similar conditions was approx. 120 kDa, which is greater than the molecular mass of free PDK3 dimer (approx. 90 kDa). This difference most likely reflects the contribution of a bound L2 domain.

Stoichiometry of PDK3–L2 complexes obtained by chromatography on Sephadex G-150 column

The tight binding between PDK3 and L2 allowed the isolation of PDK3–L2 complexes by gel filtration. This provided us with a unique opportunity to estimate the ratio between PDK3 and the bound L2 domain. To determine the stoichiometry of PDK3–L2 complexes, the protein samples corresponding to the maximum of PDK3 peak (Figure 2B) were separated on SDS/PAGE. The protein content of PDK3 and L2 was quantified using scanning densitometry of the Coomassie Blue R-250-stained gels (Figure 3). For four preparations of PDK3–L2 analysed in this manner, the ratio between PDK3 protein and L2 protein was approx. 3.2–3.7 to 1 ($n = 6$). Taking into consideration the molecular mass of PDK3 protomer (45.804 kDa) and L2 (13.797 kDa), the ratio between PDK3 protein and L2 protein would be 3.3:1 for $PDK3_2$–$L2_2$ complexes and 9.9:1 for $PDK3_2$–L2 complexes. The estimated values strongly suggest that the stoichiometry of PDK3–L2 complexes isolated by gel filtration corresponds to two molecules of L2 per one PDK3 dimer and indicates that each PDK dimer is capable of simultaneous binding of two L2 domains.

Hummel–Dreyer chromatography

The results presented in Figure 2(A) suggested that the complexes between PDK1 or PDK2 and L2 are dynamic and equilibrate on a time scale that is comparable with, or faster than, the separation

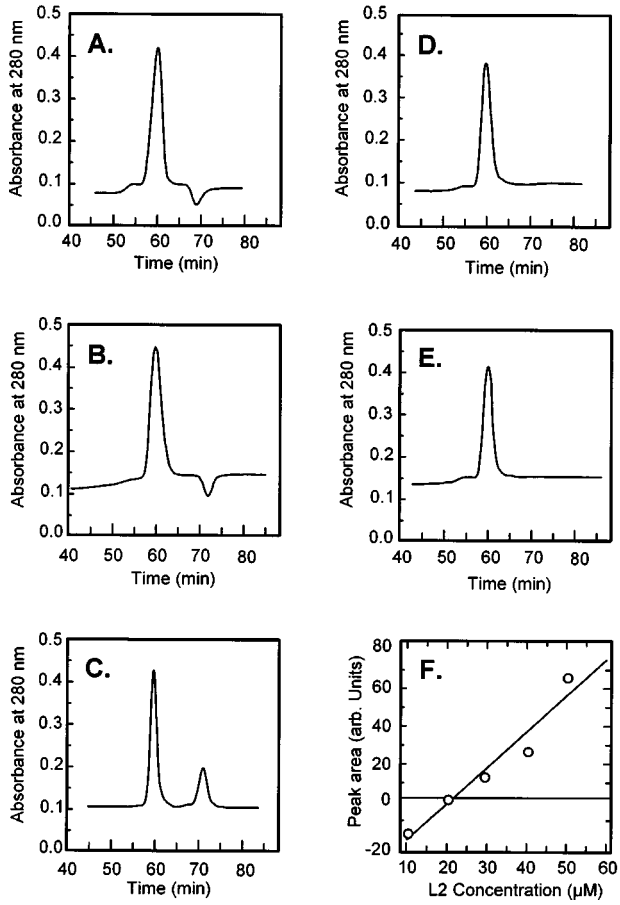

Figure 4 Hummel–Dreyer chromatography of PDK1 or PDK2 and L2 domain

A Superose 12 gel filtration column was used as described in the Experimental section. A sample of 10 μM PDK dimer and 10 μM L2 domain was applied to a column equilibrated in a buffer containing 10 μM of L2 [PDK1 (**A**); PDK2 (**B**)]. A trough at normal elution time of L2 is observed as a result of the L2 bound to PDK being eluted faster than free L2. (**C**) Approximately 10 μM PDK2 dimer and 10 μM L2 were loaded on to the column equilibrated in a buffer made without the L2 domain. The elution times of PDK2 and L2 were the same as when the proteins were applied separately to the column. A sample of 10 μM PDK dimer and 10 μM unlipoylated L2 domain was applied to a column equilibrated in a buffer containing 10 μM unlipoylated L2 [PDK1 (**D**); PDK2 (**E**)]. There was no trough observed at the normal elution time of L2. (**F**) The areas of the L2 peaks and troughs following application of various amounts of L2 (10–50 μM) and 10 μM PDK1 dimer to a column equilibrated with a buffer containing 10 μM L2 against the added L2 concentration.

time on a gel-filtration column. To estimate the strength of interaction between PDK1 or PDK2 and L2 we used the 'equilibrium chromatography' method developed by Hummel–Dreyer [28]. In a typical Hummel–Dreyer experiment, the gel-filtration column is equilibrated in a buffer containing a uniform concentration of the ligand. The protein sample containing the same concentration of the ligand is loaded on to the column. If the protein and ligand do not interact, the concentration of the ligand in the effluent remains constant throughout the run. When the protein and the ligand interact, the amount of free ligand in the applied sample is reduced by the amount bound to the protein. Bound ligand has higher mobility and is eluted from the column before the free ligand. Since the amount of free ligand is less than the column concentration of the ligand, a trough is observed at the position corresponding to the elution time of the ligand.

Figure 4 represents the results of a Hummel–Dreyer experiment under the conditions when the gel-filtration column was equilibrated in a buffer containing 10 μM L2. The samples of 10 μM PDK1 (Figure 4A) or 10 μM PDK2 (Figure 4B) supplemented with 10 μM L2 were injected on to the column. The column was developed at a flow rate of 0.2 ml/min. The effluent solution was continuously monitored with 5 mm quartz micro-flow cell by means of a UV-MII detector at 280 nm. Separation of both PDK1 and PDK2 under these conditions resulted in the appearance of a characteristic trough in the position corresponding to the position of free L2, indicative of complex formation (elution positions of free PDK2 and L2 are shown in Figure 4C). The area of trough increased as the concentration of PDKs in the applied sample increased (results not shown), reflecting the reversible nature of the interaction. Interestingly, with unlipoylated L2 used as a ligand, there were no troughs observed at the elution position of L2, when either PDK1 or PDK2 was loaded on to the column at a concentration of 10 μM (Figures 4D and 4E respectively). This suggests that the productive interaction between either PDK1 or PDK2 and L2 domain strongly depends on the proper lipoylation of the domain.

In order to estimate the strength of the interaction between PDK1 or PDK2 and the L2 domain, the concentration of free L2 in the kinase–L2 samples loaded on to the column must be known. These values were determined by applying samples with different concentrations of L2 and a constant concentration of kinase on to the column equilibrated in a buffer of a uniform concentration of L2 followed by measurement of the areas of the troughs (or peaks). The mole excess needed to fill the trough was determined by plotting the area of the trough (or peak) against the concentration of L2 in the sample buffer. The results of a typical experiment are shown in Figure 4(F). In this example, the concentration of PDK1 in applied samples was 10 μM. The concentration of L2 in the column buffer was 10 μM. The concentration of L2 in the sample was varied from 10 to 50 μM. Under the described conditions, there was an approximately linear relationship between the area of the L2 trough (or peak) and the L2 concentration in the sample buffer (Figure 4F). The plot crossed zero area at a total L2 concentration of 20 μM. Taking into account that the column was equilibrated with 10 μM L2, the concentration of L2 bound to 10 μM PDK1 dimer or 20 μM PDK1 monomer subunits can be estimated as 10 μM. Assuming two independent L2 binding sites per kinase dimer, as follows from the results of experiments presented in Figure 3, these data can be used to estimate the dissociation constant: $K_d = [PDK1][L2]/[PDK1-L2] = (20\ \mu M - 10\ \mu M)(10\ \mu M)/10\ \mu M = 10\ \mu M$. This analysis performed at different concentrations of L2 in the column buffer and different concentrations of kinase in the sample buffer yielded the average dissociation constants of $10.3 \pm 1.2\ \mu M$ ($n = 5$) for PDK1 and $10.2 \pm 2.3\ \mu M$ ($n = 5$) for PDK2. These values are in agreement with the conclusions reached based on the results of affinity chromatography.

The strong dependence of interaction between either PDK1 or PDK2 and L2 on the proper lipoylation of the L2 domain prompted us to investigate the effect of lipoylation on PDK3–L2 interaction. PDK3–L2 complexes were obtained by mixing the purified components. The resulting complexes were separated on the column equilibrated in buffer made without L2. The effluent was collected in a fraction collector. The protein composition of individual fractions was analysed by SDS/PAGE. Protein bands were visualized using silver staining. The elution profile of PDK3 reconstituted with lipoylated L2 showed the presence of L2 in PDK3-containing fractions, similar to the results presented in Figure 2(B). When unlipoylated L2 was used as a ligand, there was a partial separation of PDK3 and L2 peaks (Figure 5), suggesting that PDK3–L2 interaction is also dependent upon the proper lipoylation of L2. However, the effect of lipoylation was less dramatic than that for PDK1 and PDK2.

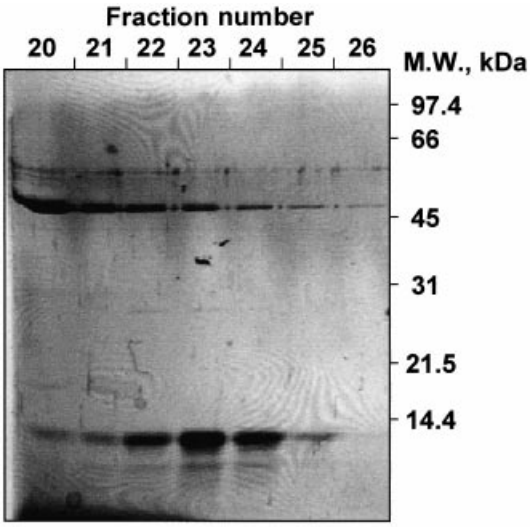

Figure 5 SDS/PAGE of fractions obtained during chromatography of PDK3 and unlipoylated L2 on Superose 12 column

Approximately equimolar amounts of PDK3 and L2 were reconstituted on ice for 30 min. Mixtures were chromatographed on Superose 12 column as described in the Experimental section. The effluent was collected in 0.2 ml fractions. Aliquots of PDK3-containing fractions were electrophoresed on SDS/PAGE (15% gel). The gel was stained with silver. The apparent molecular masses are indicated on the right-hand side.

Effect of L2 domain on enzymic activities of individual isoenzymes of PDK

In order to investigate the role of L2 binding in the regulation of kinase activity, we determined the effect of monomeric L2 domain on enzymic activities of individual isoenzymes of PDK. The experiments were performed as described in the Experimental section using 20 nM kinase, 5 μM free E1 component as a substrate and monomeric L2 domain at a final concentration of 80 μM. As a control, similar experiments were performed with human E2 component, which was used instead of the L2 domain at a final concentration of 20 nM. As shown in Figure 6, neither PDK1 nor PDK2 responded to the addition of monomeric L2 at a concentration as high as 80 μM. In contrast with PDK1 and PDK2, there was a substantial increase in PDK3 activity in response to the addition of L2. At 80 μM of L2 the activation was approx. 3-fold. Finally, there was a small, but statistically significant, increase in PDK4 activity in response to the addition of L2 domain (approx. 37%). Thus it appears that the effects of L2 domain on enzymic activities of individual isoenzymes of PDK are isoenzyme-specific. In this respect, the isoenzymes fall into two categories: PDK1 and PDK2, which do not respond to the monomeric L2, and PDK3 and PDK4, which become activated in the presence of L2. Interestingly, under the conditions described, the rate of phosphorylation of the E1 component on the addition of E2 increased 5.5- and 3.9-fold for PDK1 and PDK2 respectively and approx. 38-fold for PDK3. Addition of the E2 component also caused significant activation of PDK4 (approx. 2.3-fold). Taken together, these results indicate

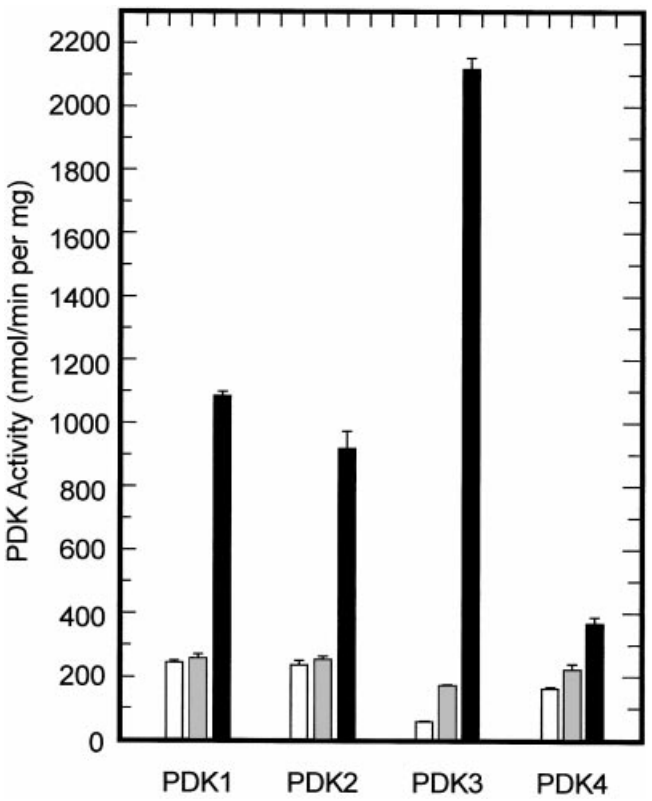

Figure 6 Effects of monomeric L2 domain and E2/E3BP component of human PDC on enzymic activities of individual PDK isoenzymes

Each reaction mixture received 5 μM E1 component, 0.4 mM ATP (specific radioactivity of 100–200 c.p.m./pmol) and 20 nM appropriate PDK in 20 mM Tris/HCl (pH 7.8), 5 mM $MgCl_2$, 50 mM KCl, 1 mM 2-mercaptoethanol. Closed bars, E2/E3BP complex used at a concentration of 20 nM; shaded bars, L2 domain used at a concentration 80 μM; open bars, experiments performed with a negative control (−effectors). Rates of phosphorylation reaction were determined after 30 s of incubation. Data are means ± S.D., $n = 9$.

that the effects of L2 and E2 on enzymic activities of individual isoenzymes of PDK are isoenzyme-specific.

DISCUSSION

Results presented here clearly demonstrate that isoenzymes of PDK are markedly different with respect to their abilities to interact with the L2 domain of the transacetylase component. PDK3 binds L2 so tightly that the respective complexes can be isolated using size-exclusion chromatography. In contrast, binding of PDK1 and PDK2 to the lipoyl-bearing domain is readily reversible, as evidenced by the results of gel filtration on Sephadex G-150 column. Using the equilibrium-binding assay developed by Hummel and Dreyer [28], the apparent dissociation constants for monomeric L2 domain were estimated to be approx. 10 μM for both isoenzymes. Taking into account the intramitochondrial concentration of PDC [5], these values are within the physiologically relevant range of concentrations.

It is interesting to note that PDK1 and PDK2 displayed greatly reduced affinity for unlipoylated L2 in Hummel–Dreyer experiments, suggesting that lipoic acid itself might be one of the major determinants recognized by these isoenzymes. By contrast, PDK3 binding was less sensitive to the proper lipoylation of L2, indicating that it can utilize binding determinants other than lipoic acid.

Using affinity purification, we were able to detect the interaction between PDK4 and L2, but the amount of PDK4 isolated on the L2 domain construct was very low. Furthermore, PDK4 did not show an appreciable interaction with L2 in Hummel–Dreyer experiments under the conditions when binding of L2 to either PDK1 or PDK2 was readily detectable (A. Tuganova, unpublished work). Taken together, these observations are consistent with the idea that PDK4 has a very low affinity for monomeric L2. This conclusion, however, will require further examination when methods for the analysis of very weak protein–protein interactions are available.

Analysis of the protein content of PDK3–L2 complexes isolated by size-exclusion chromatography revealed that the ratio between the PDK3 protein and L2 protein was approx. 3.5:1. The latter translates into the stoichiometry of 1 mol of PDK3 dimer per 2 mol of L2. In agreement with this result, the molecular mass of PDK3–L2 complexes determined by gel-filtration was approx. 120 kDa, which roughly corresponds to the molecular mass of $PDK3_2$–$L2_2$ complex. These results indicate that each kinase molecule can simultaneously bind two L2 domains. Furthermore, the binding of the first L2 apparently does not interfere with the binding of the second L2.

These conclusions are in agreement with the recently published three-dimensional structure of PDK2 [33]. This structure revealed that PDK2 is organized as a dimer with individual protomers associated in head-to-head orientation. The carboxy-terminal nucleotide-binding domains form the dimer interface. The well-isolated amino-terminal domains carrying putative lipoyl-bearing domain-binding sites extend from the interface and do not interact with each other [33]. The distances between the lipoyl-bearing domain-binding sites in this arrangement are sufficient to accommodate two L2 domains without any conformational interference.

The structural data along with the results reported here provide a support for the 'hand-over-hand' transfer hypothesis, which was proposed to explain the mechanism of PDK delivery to the multiple copies of E1 immobilized on the different edges of the E2 core [15]. According to this hypothesis, the kinase is directly transferred from one lipoyl-bearing domain to another without losing the grip of the complex. This allows a single kinase molecule to serve multiple copies of E1 without dissociation from the complex [15]. In order for this mechanism to operate properly, the kinase must be capable of binding the two lipoyl-bearing domains simultaneously during the transfer step. Data presented here show that PDK3 can bind two L2 domains simultaneously and, therefore, can be transferred along with the E2 core surface by 'hand-over-hand' mechanism. It remains to be established whether the same mechanism operates for PDK1, PDK2 and PDK4.

It is generally believed that the docking of PDK to the E2 core through the lipoyl-bearing domains largely determines the level of kinase activity. Our results show that all four isoenzymes become activated when phosphorylation reactions are supplemented with a transacetylase component. The extent of the increase in kinase activity caused by E2 decreases in the order PDK3 > PDK1 = PDK2 > PDK4. The latter correlates with the strength of the interaction with L2 domain, displayed by the individual isoenzymes (order PDK3 > PDK1 = PDK2 > PDK4) and indicates that binding to L2 is essential in mediating the effect of transacetylase. On the other hand, L2 binding by itself is clearly insufficient in maintaining the enhanced kinase function observed in the presence of E2. This conclusion is supported by our results showing that the addition of monomeric L2 has no effect on the activities of PDK1 and PDK2 and has a rather small effect on the activities of PDK3 and PDK4

when compared with the increase caused by E2 (3-fold versus 38-fold and 37 versus 130% respectively). This strongly suggests that the co-localization and mutual orientation of PDK and E1 on E2 core along with the conformational changes in the E1 structure caused by E2 binding might play an important role in supporting the enhanced kinase function.

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