Heterologously expressed inner lipoyl domain of dihydrolipoyl acetyltransferase inhibits ATP-dependent inactivation of pyruvate dehydrogenase complex

Identification of important amino acid residues

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The activity of the pyruvate dehydrogenase multienzyme complex (PDC), which catalyses the oxidation of pyruvate to acetyl-CoA within the mitochondrion, is diminished in animal models of diabetes. Studies with purified PDC components have suggested that the kinases responsible for inactivating the decarboxylase catalytic subunits of the complex are most efficient in their regulatory role when they are bound to dihydrolipoyl acetyltransferase (E2) subunits, which form the structural core of the

complex. We report that the addition of an exogenous E2 subdomain (inner lipoyl domain) to an intact PDC inhibits ATP-dependent inactivation of the complex. By combining molecular modelling, site-directed mutagenesis and biophysical characterizations, we have also identified two amino acid residues in this subdomain (Ile²²⁹ and Phe²³¹) that largely determine the magnitude of this effect.

INTRODUCTION

The pyruvate dehydrogenase multienzyme complex (PDC; 5–10 MDa) catalyses the formation of acetyl-CoA from pyruvate through the highly regulated and co-ordinated action of multiple enzyme subunits. In eukaryotes the PDC is located in mitochondria. Multiple copies of three enzymes participate in its overall catalytic action: pyruvate decarboxylase (E1), dihydrolipoyl acetyltransferase (E2) and dihydrolipoyl dehydrogenase (E3). In addition to their catalytic role, the E2 subunits perform a structural role by forming, in mammals, a pentagonal dodecahedral scaffold to which the E1 and E3 catalytic subunits bind [1]. Multiple copies of an E3-binding protein, E3BP, are also part of the complex, but their catalytic function (if any) is unknown.

The PDC is regulated by reversible phosphorylation. ATP-dependent phosphorylation of E1 serine residues leads to the inactivation of the complex; dephosphorylation re-activates it [2]. Although each E1 subunit contains three serine residues that are subject to phosphorylation by pyruvate dehydrogenase kinase (PDK), E1 inactivation is achieved by the phosphorylation of a specific serine residue [3,4]. In humans, four PDK isoenzymes [5,6] and one PDC phosphatase (PDP) isoenzyme [7] have been identified so far, and binding to the E2 subunit has been reported to be important for the efficient catalytic function of both PDK(s) and PDP [8,9].

Increased PDK and decreased PDC activities have been reported in rodent models of insulin resistance and diabetes (rats

fed on a high-fat diet; alloxan-diabetic rats) [10–12]. Although elevated PDK activity has not been reported in diabetic patients, diminished PDC activity seems to be related to the decreased rates of oxidative disposal of glucose reported in patients with Type 2 diabetes mellitus [13]. In early clinical studies, dichloroacetate (DCA), a known inhibitor of PDK(s), decreased blood glucose levels in Type 2 diabetic patients [14]. Unfortunately, the toxicity of DCA and/or its metabolites precludes its use as a therapeutic agent for long-term therapy. Nevertheless inhibition of PDK might be a therapeutic approach to the treatment of Type 2 diabetes.

An understanding of the interaction between PDK(s) and the E2 subunit could allow the structure-based design of small-molecule antagonists of this interaction that might be useful as therapeutic agents. Mammalian E2 subunits consist of four functional domains linked by flexible hinge or linker regions. At the N-terminus of the E2 subunit are two lipoyl domains (E2_{L1} and E2_{L2}, each approx. 11 kDa) containing a lipoylated lysine residue that takes part in the transacetylation catalysed by the subunit [15]. The E2_{L2} domain has been shown not only to be important for catalysis but also to be involved in the binding of PDKs and PDP (isolated from bovine kidney) [8,9]. Moreover the PDKs can be transferred from a PDC subcomplex containing an intact bovine E2 core to a heterologously expressed human E2_{L2} domain [8]. In contrast with previous studies, which have analysed the interaction between PDK and E2_{L2} by using kinase purified from bovine kidney and either native or heterologously expressed E2, we have employed site-directed mutagenesis of

Abbreviations used: DCA, dichloroacetate; E1, pyruvate decarboxylase; E2, dihydrolipoyl acetyltransferase; E3, dihydrolipoyl dehydrogenase; MD, molecular dynamics; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PDP, PDC phosphatase; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; rE2_{L2}, recombinant E2_{L2}; rPDK, recombinant PDK.

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human recombinant E2_{L2} (rE2_{L2}) in an attempt to define further the interaction between PDK and E2_{L2} within the context of an intact PDC.

EXPERIMENTAL

Materials

The GCG sequence alignment suite of programs was purchased from Genetics Computer Group (Madison, WI, U.S.A.). The molecular modelling package SYBYL was purchased from Tripos Associates (St. Louis, MO, U.S.A.). Cloning and expression vectors were purchased from Novagen Inc. (Madison, WI, U.S.A.). The QuickChange[™] site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, U.S.A.). Chromatography materials were from Pharmacia (Uppsala, Sweden). Preparations of pig PDC containing intrinsic kinase activity were purchased from Sigma (St. Louis, MO, U.S.A.; catalogue numbers P-7032 and P-5194). The N-acetylated tetradecapeptide Ac-YHGHMSDPGVSYR was synthesized by Genosys Biotechnologies (The Woodlands, TX, U.S.A.). Sodium DCA was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). [γ -³²P]ATP was obtained from DuPont Life Science Products (Boston, MA, U.S.A.; catalogue number NEG302H). Laemmli buffer, an SDS/PAGE denaturing reagent, was purchased from Sigma. Electrophoretic equipment, precast Tris/glycine Ready Gels[™] and concentrated SDS/PAGE buffers were purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). The following visualization equipment and software were purchased from Molecular Dynamics (Sunnyvale, CA, U.S.A.): Phosphor screen, Storm 840 Imager[™], Personal Desitometer ST[™] and Image QuANT[™].

Modelling of human E2_{L2}

Modelling of the structure of the human E2_{L2} exploited the high sequence similarity with lipoyl domains of other organisms. The multiple sequence alignment of the lipoyl domains reported by Dardel and co-workers [16] was used as a starting point for our analysis with the GCG suite of programs. The basis of the homology model was the published NMR solution structure of *Azotobacter vinelandii* [17]. The human E2_{L2} was then specifically aligned with respect to the *A. vinelandii* sequence by using the BESTFIT algorithm of GCG. Comparison of the BESTFIT alignment with the consensus sequence alignment revealed no significant differences. The co-ordinates for the human E2_{L2} were derived from the *A. vinelandii* sequence by using the Composer model of SYBYL [18–20].

All of the modelling was performed with the AMBER all-atom forcefield and Gasteiger charge assignment methods implemented in SYBYL. A 10 Å cut-off was employed for all non-bonded interactions. Molecular dynamics (MD) simulations were performed primarily to equilibrate the solvent around the protein rather than to derive an ensemble averaged structure or an equilibrium structure. Major details of the MD simulation include a constant temperature, volume and number of particles (TVN) ensemble, a temperature of 300 K, periodic boundary conditions with a rectangular box and a time step of 1 fs. Solvation was performed by employing the TIP3P water model with at least two solvation layers. Simulations were monitored for stability on the basis of total energy, potential energy, temperature and root-mean-square fluctuations. The newly generated structure was solvated in a box of water; with the protein frozen, the water was first equilibrated around the protein by using MD. Then, with backbone atoms restrained, the protein was minimized for 100 steps of conjugate gradient

method, followed by MD. The structure at the end of the first simulation was then taken and positional restraints were placed on only the α -carbon atoms; MD was then performed for 50 ps. The structure with the lowest potential energy during this MD simulation was then chosen and minimized with no constraints or restraints in the presence of water. The resulting model served as the putative model of the human E2_{L2}.

Cloning, site-directed mutagenesis and heterologous expression of proteins

rE2_{L2} proteins

A cDNA fragment encoding the E2_{L2} domain of human PDC (residues 181–276) was isolated from human skeletal muscle mRNA by reverse transcriptase-mediated PCR and subsequently cloned into the bacterial expression vector pET17b, which directed expression of the domain with a FLAG-epitope sequence fused to its N-terminus.

Several amino acids (Val¹⁸⁸, Leu¹⁸⁹, Ser¹⁹⁴, Met¹⁹⁷, Ile²²⁹ and Phe²³¹) were initially changed singly to alanine (V188A, L189A, S194A, M197A, I229A and F231A) (see Figure 1); a double mutant, I229A/F231A, was also constructed. Mutations were constructed by PCR [21] or the QuickChange[™] site-directed mutagenesis kit and were verified by DNA sequencing with an automated DNA sequencer (ABI). Wild-type E2_{L2} and mutant rE2_{L2} proteins were expressed and purified from *Escherichia coli* as follows. Transformed *E. coli* cells were grown in the presence of 10 μ g/ml lipoic acid. Cell pellets were lysed in 50 mM Tris/HCl, pH 8.0, containing 5 mM DTT, 5 mM EDTA and 5 mM benzamidine/HCl. Clarified lysates were then loaded on a column of Q-Sepharose HP[™] pre-equilibrated with lysis buffer. rE2_{L2} was eluted with a linear gradient of 0–1 M NaCl in lysis buffer. The rE2_{L2}-containing fraction was adjusted to 40% (w/v) (NH₄)₂SO₄ and then centrifuged; the supernatant was applied to a column of Phenyl Sepharose HP[™], equilibrated with PBS/5 mM dithiothreitol [containing 40% (w/v) (NH₄)₂SO₄]. Lipoylated and non-lipoylated rE2_{L2} were eluted with a linear gradient of 100–0% (w/v) (NH₄)₂SO₄ in PBS/5 mM dithiothreitol. Lipoylated rE2_{L2} was eluted 5–10% later in the gradient than non-lipoylated material, which was a baseline separation. rE2_{L2} was then concentrated by ultrafiltration. Residual contaminating *E. coli* proteins and aggregates were removed with a Superdex 75[™] chromatography column pre-equilibrated with PBS/5 mM dithiothreitol.

rPDKs

N-terminal hexahistidine-tagged rat PDK1 and PDK2 cDNA constructs (in pET28A) were expressed in *E. coli* [strain BL21(DE3) pLysS] with the chaperones GroES/L. Clarified cell lysates (in PBS) were loaded on an XK26/10 column packed with Pharmacia Chelating Sepharose Fast Flow[™] equilibrated with PBS containing 0.5 M NaCl (buffer I) at a flow rate of 15 ml/min. The column was washed with buffer I until the A₂₈₀ of the eluate had stabilized. PDK protein was eluted with a 0–100% gradient of buffer II (buffer I plus 250 mM imidazole, pH 7.5) over 10 column volumes. rPDK-containing fractions were found at the very end of the imidazole gradient, eluting just after the main contaminating bacterial proteins. SDS/PAGE analysis revealed that the purity of each kinase form was of the order of 80%. To increase the stability of the kinases, the nickel-chelate eluates were subjected to immediate desalting into 50 mM Hepes buffer, pH 7.4, containing 100 mM (NH₄)₂SO₄ and 2 mM dithiothreitol. For speed and efficiency, an XK50/10 column of Sephadex G-25 superfine was used at 30 ml/min, enabling 30 ml

aliquots of kinase to be buffer-exchanged in less than 10 min. The desalted proteins were then concentrated approx. 5-fold by using a 30 kDa cut-off ultrafiltration membrane. The final yields were 4.8 and 4.5 mg/l for rPDKs 1 and 2 respectively.

CD spectroscopy

CD spectra were recorded on a JASCO 720 spectropolarimeter at 23 ± 0.5 °C. The sample chamber was constantly flushed with nitrogen. The instrument was calibrated with (1R)-(-)-10-camphorsulphonic acid, ammonium salt. Far-UV CD spectra were recorded from 190 to 260 nm in a 0.2 cm quartz cuvette with 0.1 nm intervals. The scanning conditions were: sensitivity 50 mdeg; bandwidth 1.0 nm; response time 0.125 s; scan speed 200 nm/min. The near-UV CD spectra were measured in a 0.2 cm quartz cuvette from 250 to 320 nm. The scan conditions were as above. Each spectrum was the average of ten (far-UV) or five (near-UV) scans and was corrected by subtracting the spectra of the appropriate buffer controls.

NMR spectroscopy

NMR spectra were recorded and analysed as previously described for the wild-type human rE2_{L2} domain [22]. Wishart plots were generated by standard procedures [23].

Design of functional PDK assay

PDK activity was assessed indirectly by measurement of residual PDC activity after kinase reaction in an assay that is similar to one that has been used to profile PDK inhibitors [24]. The assay consisted of three steps.

Step 1: preincubation/acetylation

This step was routinely included in the assay because we found that it enhanced the intrinsic PDK activity of commercially available PDC by up to 2.7-fold, presumably by increasing the acetylation status of the E2 domains [25]. Pig PDC containing intrinsic kinase activity was incubated for 40 min at 37 °C in buffer A [40 mM Mops (pH 7.20)/0.5 mM EDTA/30 mM KCl/1.5 mM MgCl₂/0.25 mM acetyl-CoA/0.05 mM NADH/2 mM dithiothreitol/10 mM NaF] at a concentration of approx. 80 µg/ml.

Step 2: PDK reaction

The PDK reaction was performed in a total volume of 100 µl at 37 °C, and was initiated by dilution of the PDC mixture 2.2-fold in 1.8 × buffer A containing 55 µM ADP, 100 µM ATP and the additions (e.g. rE2_{L2} domains) indicated (ATP was omitted in control reactions representing complete PDK inactivation or when the affect of the additions on PDC activity directly was investigated). After 3 min the kinase reaction was terminated by the addition of 0.1 vol. of stopping buffer (55 mM ADP/55 mM pyruvate).

Step 3: residual PDC activity

The PDC activity remaining was assayed in a total volume of 200 µl at 37 °C by addition of 0.82 vol. of buffer B [120 mM Tris (pH 7.8)/0.61 mM EDTA/0.73 mM MgCl₂/2.2 mM thiamine pyrophosphate/11 mM 2-mercaptoethanol/2.2 mM NAD⁺/2.2 mM pyruvate/1.1 mM CoA] by measuring the production of NADH at 340 nm. The NADH-related change in absorbance at 340 nm (Δ_{ATP} for incubations containing ATP; Δ_{noATP} for control reactions from which ATP was absent; $\Delta_{\text{ATPaddition}}$ for incubations

containing ATP plus additions) was the measure of residual PDC activity. PDK inhibition was calculated as follows:

$$\text{inhibition (\%)} = 100[(\Delta_{\text{ATPaddition}} - \Delta_{\text{ATP}})/(\Delta_{\text{noATP}} - \Delta_{\text{ATP}})]$$

Theoretical considerations of functional PDK assay

Comparisons of the effects of the various rE2_{L2} additions were made within individual experiments. The ATP-dependent inactivation of PDC approximated quasi-first-order reaction kinetics (mean ± S.E.M. estimates of rate constants were 0.0016 ± 0.00015 and 0.00058 ± 0.00026 s⁻¹ for Sigma PDC preparations P-7032 and P-5194 respectively; $n = 3$ independent experiments), and the PDC reaction was linear with time throughout step 3 (see Figure 4A). The specific activity of the PDC preparations ranged from 160 to 250 nmol/min per mg of protein for the BSA-stabilized preparation of PDC (Sigma P-7032) and from 610 to 1270 nmol/min per mg of protein for the PDC preparation without added BSA (Sigma P-5194). When ATP was included in step 2, PDC activity was inactivated by 40–74%. Thus substantial and varying degrees of substrate conversion (PDC inactivation) were obtained, which compromised data analysis by the Michaelis–Menten model. However, a simplistic theoretical analysis indicated that if inactivation of the PDC by the endogenous kinase followed quasi-first-order kinetics, and inhibition was proportional to $[1/(1 + i/K_i)]$, where i is the inhibitor concentration, i.e. if the PDC enzyme concentration is much less than the kinase K_m and simple kinetics applies, then IC₅₀ values for inhibitors would be expected to deviate from their K_i values by less than 3-fold even under conditions where substrate conversion attained 90% in control incubations. This analysis has been verified for the known PDK inhibitor 2,2-dichloro-4'-methylacetophenone (see Figures 4B and 4C).

Protein phosphorylation assay

Endogenous kinase activity

Direct assessment of PDK activity in the PDC preparation was made by measuring the initial rate of phosphorylation of the E1 PDC subunit protein by a modification of a method described by Cate and Roche [25] as follows. PDC was preincubated/acetylated as in the functional PDK assay (see step 1 above), except that the PDC concentration was 100 µg/ml in a volume of 0.45 ml. The kinase reaction was similar to the functional PDK assay (step 2), except that the reaction was initiated by the addition of [γ -³³P]ATP (80 mCi/mmol) and the final reaction volume was 1 ml. The reaction was terminated after 45 s by the addition of 1 ml of 12% (w/v) trichloroacetic acid. To minimize the loss of protein during the following manipulations, 50 µl of a 20% (w/v) solution of BSA was added. An additional 10 ml of 6% (w/v) trichloroacetic acid was added to dilute the remaining [γ -³³P]ATP, and the mixture was centrifuged at 3500 g for 15 min at 10 °C. The protein pellet was recovered, dissolved in 1 ml of 1 M NaOH and reprecipitated by the addition of 5 ml of 12% (w/v) trichloroacetic acid. Water (5 ml) was added and the mixture was recentrifuged. The pellet was recovered, redissolved, reprecipitated and centrifuged in this manner twice more. The final pellet was dissolved in 1 ml of 1 M NaOH. The radioactivity of the entire sample was determined by liquid-scintillation counting.

Activity of heterologously expressed kinases

Acetylated pig heart PDC (114 µg/ml) was incubated in a total volume of 100 µl in the presence or absence of recombinant PDK

(rPDK) (100 $\mu\text{g/ml}$) in a buffer containing 40 mM Mops, pH 7.2, 0.25 mM acetyl-CoA, 0.05 mM NADH, 30 mM KCl, 0.5 mM EDTA, 1.5 mM MgCl_2 , 2 mM dithiothreitol, 10 mM NaF and 200 μM [γ - ^{33}P]ATP (0.025 $\mu\text{Ci/nmol}$ of ATP) for 40 min at 37 °C. Phosphorylation was terminated by the addition of 100 μl of Laemmli buffer and heating to 95 °C for 10 min. Portions of the sample were separated by electrophoresis; phosphorylated proteins were revealed with a phosphor screen and a Storm 840 Imager[®].

rPDK peptide phosphorylation assay

rPDK activity was determined with a 96-well microtitre plate format. The rPDK-catalysed incorporation of [^{33}P]phospho groups into an acetylated tetradecapeptide substrate, Ac-YHGHMSDPGVSYR, was assayed at 37 °C as follows: a 45 μl reaction mixture contained peptide substrate (0.56 mM or as indicated otherwise), Mops (44 mM, pH 7.2), KH_2PO_4 (22 mM), EDTA (0.56 mM), MgCl_2 (2 mM), KCl (33 mM), dithiothreitol (2.2 mM), NaF (11 mM), rPDK (1 or 2; 53 $\mu\text{g/ml}$, or as indicated otherwise), and other additions as indicated. The reaction was initiated by adding 5 μl of 2 mM [γ - ^{33}P]ATP (0.05–0.1 $\mu\text{Ci/nmol}$ of ATP). The reaction was terminated after 30 min by the addition of 5 μl of a mixture of 425 mM H_3PO_4 and 5 mM adenosine 5'-[β , γ -imido]triphosphate (p[NH]ppA). A 20 μl portion of the acidified mixture was applied directly to the surface of phosphocellulose units, and unbound protein and free [γ - ^{33}P]ATP were removed by using the vacuum manifold system. The phosphocellulose unit was rinsed with 2 ml of 75 mM H_3PO_4 , and ^{33}P -labelled peptide was then eluted from the unit with 500 μl of 1 M NaOH. The eluted phosphorylated peptide was collected into a 1.1 ml plastic test tube, which was then transferred to a 7 ml scintillation vial. Radioactivity in the sample was determined after the addition of 5 ml of Dupont NEN-989 liquid-scintillation fluid. The results are expressed either as specific activity (nmol of phosphate transferred/mg of kinase protein per 30 min) or as a percentage of control.

RESULTS

E2_{L2} model and choice of mutants

The amino acids that were mutated were chosen by using a molecular model of the human E2_{L2} domain (amino acid residues 181–276) that was based on the recently solved NMR solution structures of bacterial PDC E2 lipoyl domains (Figure 1) [16,20,26]. As modelled, the human lipoyl domain can be viewed as a distorted and flattened β -barrel that resembles a glove with the lipoyl-lysine located at the apex of a type I β -turn, at the tip of the thumb position (Figure 1). The model also predicts a putative hydrophobic pocket on the side of the flattened β -barrel into which the lipoyl-lysine is predicted to project. This pocket contains at its perimeter six sequentially discontinuous amino acid residues (Val¹⁸⁸, Leu¹⁸⁹, Ser¹⁹⁴, Met¹⁹⁷, Ile²²⁹ and Phe²³¹) (Figure 1). Because previous reports indicate that resolved PDK(s) has exposed hydrophobic patches, and the lipoylation of Lys²²⁶ is reported to be important for E2_{L2}/PDK binding [8], we reasoned that the interaction between PDK(s) and E2_{L2} might be primarily hydrophobic in nature and might involve this putative hydrophobic pocket. For these reasons we initially chose to mutate Val¹⁸⁸, Leu¹⁸⁹, Ser¹⁹⁴, Met¹⁹⁷, Ile²²⁹ and Phe²³¹ to alanine (V188A, L189A, S194A, M197A, I229A and F231A); a double mutant, I229A/F231A, was also constructed. They were changed to alanine to preserve the overall hydrophobicity of the region, to limit or eliminate the potential for new hydrogen-bond

Figure 1 Molecular model of human E2_{L2} domain (residues 181–276)

Amino acids chosen for site-directed mutagenesis are listed in green and depicted in space-filling form. The lysine residue that is lipoylated (Lys²²⁶) is shown for reference in purple and is located at the top of the model.

formation and to minimize the likelihood of introducing major structural alterations into the rE2_{L2} domain.

Biophysical characterization of wild-type and mutant rE2L2 domains

Once the proteins had been expressed and purified, several physical techniques were used to determine the extent of lipoylation and the folding of the purified wild-type and mutant domains.

First their molecular masses were determined by electrospray MS. The purified lipoylated forms of the domains were fully lipoylated (results not shown; the wild-type domain was also expressed in non-lipoylated form). Thus none of the mutations eliminated the ability of the domains to be post-translationally lipoylated.

As a confirmation of structure, lipoylated wild-type and mutant domains were analysed by UV CD spectroscopy (Figure 2). With the exception of the I229A/F231A and V188A mutant proteins, the mutant proteins exhibited far-UV CD spectra that were similar to that of the wild-type lipoylated protein (Figure 2A). Calculations with SELCON [27] predicted these proteins to contain 5–6% α -helical, 41–43% β -sheet and 24–27% β -turn structural motifs, which corresponds well to our model. The main structural feature of the V188A and double-mutant proteins was also β -sheet, and there was only a small change in the

Figure 2 Far-UV and near-UV CD spectroscopy of wild-type and mutant lipoylated rE2_{L2} proteins

(A) The far-UV spectra reveal that the wild-type and mutant rE2_{L2} domains are predominately β -sheet structure. (B) The near-UV spectra demonstrate that the lipoylated wild-type and mutant proteins all contain a buried tryptophan residue that is in a similar environment.

Figure 3 Wishart plots of the wild-type (A), I229A (B) and F231A (C) rE2_{L2} proteins

Wishart plots for the wild-type and I229A proteins (A,B) are identical. Differences from wild-type observed in the F231A mutant protein are marked with triangles.

predicted α -helical content (7% for V188A and 3% for the double mutant).

The near-UV CD spectra of the lipoylated wild-type and mutant proteins were also compared. The UV CD spectra between 280 and 300 nm reflected the chirality of a single tryptophan residue present in each of these proteins. The wild-type and mutant proteins exhibited similar near-UV CD spectra (Figure 2B), suggesting that this tryptophan residue was buried and similarly constrained by adjacent residues in all of the proteins. Thus both the far-UV and near-UV CD spectra support the conclusion that the secondary structures and folding of the mutant proteins were not grossly altered from those of the wild-type protein.

The Wishart plots of the α -proton chemical shifts of the wild-type, F231A and I229A rE2_{L2} mutant predict similar secondary structures for this region (Figure 3). Indeed, the Wishart plots of the entire wild-type and I229A rE2_{L2} mutant proteins were superimposable. In the Wishart plot of F231A mutant protein only five differences were observed from wild-type (Leu¹⁹⁰, Ala¹⁹², Leu²¹⁸, Val²³³ and Leu²³⁹), which can be ascribed to loss of interactions with the aromatic side chain of Phe²³¹ (Figure 3).

Validation of the functional PDK assay

The PDC reaction was linear throughout step 3 of the PDK functional assay, as shown in Figure 4. The theoretical analysis of the assay was verified with the PDK inhibitor 2,2-dichloro-4'-methylacetophenone. This compound has been reported to exhibit a K_i of 1 μ M, on the basis of its effect on the first-order rate constant for the ATP-dependent inactivation of purified beef heart PDC containing intrinsic PDK activity [24]. We determined the IC₅₀ of this compound in our functional PDK assay, and its K_i in a modified version of the functional assay (different kinase reaction times) that allowed us to examine its effect on the first-order rate constant of the ATP-dependent

Figure 4 Validation of the functional PDK assay

(A) The pyruvate dehydrogenase reaction was linear throughout step 3 (values are means $\pm$ S.E.M. for three independent experiments). (B) The IC_{50} value for 2,2-dichloro-4'-methylacetophenone (DMP) in the functional assay was determined as $3.1 \pm 0.52 \mu\text{M}$ (values are means $\pm$ S.E.M. for four independent experiments). (C) The effect of DMP on the first-order rate constant of PDK(s) was determined in a modified version of the functional PDK assay in which the kinase reaction time was varied from 45 s to 7 min. The K_i value for DMP was determined as $2.6 \pm 0.81 \mu\text{M}$ (values are means $\pm$ S.E.M. for four independent experiments).

Figure 5 Heterologously expressed human rE2L₂ inhibits PDK activity in a pig PDC preparation

(A) Lipoylated wild-type rE2L₂ and mutants V188A, L189A, S194A and M197A all inhibited PDK activity in a dose-dependent manner, whereas non-lipoylated wild-type rE2L₂ had essentially no effect. (B) Lipoylated mutants I229A, F231A and double-mutant I229A/F231A showed a marked decrease in the ability to inhibit PDK activity (compared with wild-type). The values of percentage inhibition of PDK are shown as means $\pm$ S.E.M. for at least three independent experiments except for M197A, for which values are shown as means $\pm$ range for two independent experiments. (C) Wild-type rE2L₂ exhibited identical inhibitory effects on PDK function in BSA-stabilized and BSA-free preparations of PDC. Values shown are means $\pm$ S.E.M. for three independent experiments.

Figure 6 Effect of wild-type lipoylated and non-lipoylated heterologously expressed human rE₂_{L2} proteins on the ability of PDK to phosphorylate pig PDC E1 protein

Symbols: ▲, non-lipoylated rE₂_{L2} protein; ▼, lipoylated rE₂_{L2} protein. The results are means $\pm$ S.E.M. for three or four independent experiments.

inactivation of PDC. These analyses produced IC₅₀ and K_i values of 3.1 ± 0.52 and 2.6 ± 0.81 μ M, respectively (mean $\pm$ S.E.M. for four independent experiments).

Effects of rE₂_{L2} proteins in the PDK functional assay

We evaluated the mutant rE₂_{L2} proteins in an assay based on a commercial preparation of pig PDC containing intrinsic PDK activity (the PDK isoenzyme composition is unknown). The assay for PDK activity consisted of three steps: the first step was a preincubation step allowing the intrinsic PDK activity of the complex to be increased; in the second step, PDC was inactivated in the presence of ATP; the PDC activity remaining was determined in the third step. Because previous work demonstrated that the E₂_{L2} domain can bind PDK(s) [8], it seemed plausible that addition of this domain could alter PDK function in this assay.

Wild-type lipoylated rE₂_{L2} inhibited the ATP-dependent inactivation of BSA-stabilized PDC in a dose-dependent manner up to 78 % at a concentration of 120 μ g/ml (approx. 10 μ M) (Figure 5A). Similar results were obtained with a preparation of PDC of higher specific activity that was not stabilized with BSA (Figure 5C). Support for this inhibitory effect being predominantly due to the inhibition of PDK and not the activation of PDC was provided by the finding that lipoylated rE₂_{L2} had no effect on PDC function in the absence of ATP at the concentrations tested (results not shown). In comparison, DCA inhibited PDK activity by only 20 % at a concentration of 1 mM (results not shown). This low efficacy of inhibition by DCA might reflect the PDK isoenzyme(s) composition of the PDC preparations used, because 1 mM DCA inhibited rPDKs 1 and 2 by 57 % and 23 % respectively (see Figure 7C). The finding that non-lipoylated

Figure 7 Inhibition of heterologously expressed rat PDKs 1 and 2 by p[NH]ppA and DCA

Lineweaver-Burk plots revealed that p[NH]ppA exhibited competitive inhibition with respect to ATP (A, B). rPDKs 1 and 2 exhibited differential susceptibility to inhibition by DCA (C). Results are expressed either as means of triplicate determinations from a single experiment (A, B) or as means $\pm$ S.E.M. from three independent experiments (C).

Figure 8 Phosphorylation of E1 protein by rPDK2 and autophosphorylation of rPDK2

Phosphorylation of E1 by PDK present in the PDC preparation (lane labelled PDC) is increased by the addition of rPDK2 (lane labelled PDC + rPDK2). rPDK2 is subject to autophosphorylation (lane labelled rPDK2). Results shown are from a representative experiment. The molecular masses of protein standards (lane Std) are shown at the left.

rE2_{L2} had no effect on PDK activity (Figure 5A) is consistent with previous reports demonstrating the importance of the lipoyl group for the interaction of E2_{L2} and PDK [8]. The lipoylated L189A, S194A and M197A mutant proteins had similar inhibitory effects to wild-type rE2_{L2} on PDK activity, whereas the V188A mutant protein exhibited a modest decrease in the ability to inhibit PDK activity that might be due to the change in its structure (Figure 2A) (55% compared with 75% for the wild-type at 100 µg/ml) (Figure 5A). In contrast, mutating Ile²²⁹ or Phe²³¹ to alanine decreased the ability of the respective mutant domains to inhibit PDK activity (Figure 5B). At a concentration of 120 µg/ml the I229A and F231A mutant proteins inhibited PDK activity by only 38% and 48% respectively (Figure 5B). The lipoylated double mutant (I229A/F231A) exhibited the least ability to inhibit PDK activity (maximum inhibition of 28% at 120 µg/ml) (Figure 5B).

Effect of wild-type rE2_{L2} on endogenous PDK phosphorylation of E1

The phosphorylation of E1 by PDK was inhibited by lipoylated wild-type rE2_{L2} in a dose-dependent manner up to 76% inhibition at a concentration of 120 µg/ml (approx. 10 µM) (Figure 6). In contrast, the non-lipoylated wild-type protein exhibited a modest, but non-significant, biphasic increase in E1 phosphorylation.

Catalytic activity of rPDKs

The rPDKs were catalytically active, as demonstrated by the peptide phosphorylation assay. The catalytic activity of rPDKs 1 and 2 was inhibited both by p[NH]ppA, a non-hydrolysable ATP analogue, and by DCA (Figure 7). p[NH]ppA exhibited competitive inhibition with respect to ATP for both kinase isoenzymes (Figures 7A and 7B). The *K_i* values for p[NH]ppA were estimated from the slopes of Lineweaver–Burk plots (approx. 80 and approx. 40 µM for PDK1 and PDK2 re-

spectively). E1 also served as a substrate for rPDK2; interestingly, we demonstrated that rPDK2 is subject to autophosphorylation (Figure 8) (rPDK1 was not tested).

DISCUSSION

We have explored the effect of the rE2_{L2} domain on PDK function within the context of an intact PDC. However, the functional and E1 phosphorylation assays employed in these studies did not allow us to explore fully the mechanism(s) by which the lipoylated wild-type rE2_{L2} domain inhibited the ATP-dependent inhibition of PDC. The interaction of PDK with PDC is complex, and the ratio of the heterologously expressed rE2_{L2} proteins to PDC components (including endogenous PDK) was not established. To explore the mechanism further, we attempted to simplify the interaction by developing a kinase/rE2_{L2} binding assay based on heterologously expressed proteins. Despite evidence that PDKs resolved from bovine kidney bind human lipoylated E2 and E2_{L2} domains [8], we demonstrated no affinity between wild-type lipoylated or non-lipoylated rE2_{L2} and the heterologously expressed, catalytically active rat kinases in surface plasmon resonance studies [28,29] (results not shown). It is noteworthy in this regard that, although four isoenzymes of PDK have been cloned in the last four years, the only evidence so far that heterologously expressed kinase interacts with E2 is indirect and comes from studies examining the effect of such an association on kinase activity [30]. These findings suggest that the binding of PDKs to E2 might require post-translational modification of PDK(s) and/or that the binding of PDK(s) to E2 is via a complex binding stoichiometry (e.g. heterodimerization of PDKs).

The possibility that the rE2_{L2} domain inhibited PDK activity indirectly by binding to the E1 subunits of PDC and thereby blocking their phosphorylation seems unlikely. First, the rE2_{L2} domain did not contain amino acid residues that are known to be required for E1 subunit binding [31]. Secondly, the interaction of the E2 lipoyl domain with the E1 subunit has been reported to be independent of its lipoylation status [32]. The ability of the rE2_{L2} domain we expressed to inhibit PDK function was dependent on its lipoylation. Therefore, if E1 binding occurred, PDK inhibition would have been entirely due to the presence of lipoyl groups. This seems improbable because chemical modification of the lipoyl group has been shown to be without effect on the ability of this domain to bind PDK(s) [8].

It is more plausible that the heterologously expressed rE2_{L2} domain interacted directly with PDK(s), because resolved PDK has been reported to bind to lipoylated, but not non-lipoylated, E2 and E2_{L2} [8]. The heterologously expressed rE2_{L2} domains are unlikely to have served as structural components of PDC because they exhibited no effect on pyruvate dehydrogenase activity at the concentrations tested. Inhibition of kinase function could therefore have been achieved by an association of free lipoylated rE2_{L2} with PDK(s) that changed the equilibrium between free PDK(s) and PDK(s) bound to PDC.

As is true of many protein–protein interactions, hydrophobic interactions and side-chain packing might have had a role in the ability of the lipoylated rE2_{L2} domain to inhibit PDK function in our studies. Two residues, Ile²²⁹ and Phe²³¹, and perhaps to a smaller extent also Val¹⁸⁸, seem to have largely determined the magnitude of the effect, because the overall hydrophobicity and secondary structures of the mutant rE2_{L2} domains were preserved. None of the mutations completely eliminated the ability of the rE2_{L2} domain to inhibit PDK activity in our assay; therefore this domain might have inhibited PDK(s) through one or more less specific hydrophobic interactions and/or an interaction entirely

dependent on the presence of the lipoyl group on the rE2_{L2} domain. Additionally, residues outside the putative hydrophobic pocket might have had a role in the ability of the domain to inhibit PDK function. The double mutant, I229A/F231A, underlines the importance of Ile²²⁹ and Phe²³¹ in determining the magnitude of the effect.

The ability of rPDK2 to undergo autophosphorylation raises several questions. Are native PDKs also subject to phosphorylation? If so, what are the consequences of phosphorylation on PDK activity? Are phosphorylated PDKs dephosphorylated by one or more specific phosphatases?

In summary, we have identified amino acid residues on the rE2_{L2} domain that are important determinants in its ability to inhibit PDK function in the context of the PDC. These findings raise the possibility that these amino acid residues have a role in the interaction between the E2 subunit and this regulatory kinase(s), which in part regulates the function of a multienzyme complex with a key role in intermediary metabolism.

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