Regulation of mammalian pyruvate dehydrogenase kinase

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Abstract It is generally believed that mammalian pyruvate dehydrogenase kinase is a heterodimer consisting of catalytic and regulatory subunits. However, the contribution of the two subunits to the kinase-mediated signal transduction has remained undefined. In the present study recombinant components of mammalian pyruvate dehydrogenase complex were employed in order to characterize the role of the kinase catalytic subunit in the regulation of pyruvate dehydrogenase reaction. The results provide the first evidence strongly suggesting that the catalytic subunit of pyruvate dehydrogenase kinase is competent to respond to known effectors of kinase activity as well as to interact with the E_2-core without assistance of a regulatory subunit.

Key words: Pyruvate dehydrogenase complex; Pyruvate dehydrogenase kinase; Regulation; Metabolism of carbohydrates

1. Introduction

Pyruvate dehydrogenase kinase (PDK) catalyzes phosphorylation of the mitochondrial pyruvate dehydrogenase complex (PDC) that leads to its complete inactivation. By this means, PDK serves as an efficient tool to control flux through the complex and, in turn, the rate of aerobic oxidation of carbohydrates (for review see [1–3]). The enzymatic activity of PDK is regulated by an array of compounds that are substrates, products and co-factors of the pyruvate dehydrogenase reaction. As a general rule, the products of dehydrogenase reaction activate, while the substrates inhibit PDK activity. This allows the kinase to sense the metabolic need for carbohydrates catabolism and accordingly adjust the activity of PDC [1]. The exact molecular mechanisms that are employed by most of the effectors in order to regulate kinase activity have not been elucidated entirely. It is believed that only pyruvate inhibits PDK activity directly via binding to a specialized allosteric site on the kinase molecule [4,5]. The effects of NAD^+, NADH, CoA and acetyl-CoA, in contrast, have been proposed to be indirect [6], mediated by the lipoyl-prosthetic groups covalently attached to the PDC core [7].

Structurally, PDK purified from bovine kidney appears to consist of two non-equivalent subunits with molecular weights of 48 000 and 45 000 Da [8]. Elegant experiments with selective digestion of the 48 000 Da subunit by chymotrypsin and the 45 000 Da subunit by trypsin established that the catalytic function of PDK is associated with the 48 000 Da subunit. The function of the 45 000 Da subunit has not been determined, but it was suggested that it may be responsible for targeting of the catalytic subunit to the complex and/or for the allosteric regulation of the kinase activity [8]. The evidence for a regulatory subunit has made the status of PDK's catalytic subunit uncertain for want of data on whether it can interact with the PDC core and also respond to physiologically relevant effectors in the absence of the regulatory subunit.

Recently, this laboratory made considerable progress in the molecular cloning and expression of cDNAs encoding isoenzymes of the catalytic subunit of PDK [9]. This, along with recent success in the expression and reconstitution of the PDC from recombinant proteins [10], allowed me to take another look at the regulation of PDK activity using completely defined, recombinant components of the mammalian PDC. The present manuscript reports the effects of different components of PDC on the enzymatic activity of the recombinant catalytic subunit of PDK along with the first evidence that the catalytic subunit itself is competent to respond to effectors of PDK activity.

2. Materials and methods

2.1. Expression and purification of rat PDK

The molecular cloning of the cDNA encoding for the rat isoenzyme 2 of PDK (RPDK2) was reported previously [11]. The construction of the bacterial expression vector based on pET-28a (Novagen) was reported in [9]. Expression and purification of the His-tagged RPDK2 was performed essentially as described elsewhere [9] with some modifications. Briefly, the expressing *E. coli* strain was allowed to grow at 37°C in M9ZB media containing 45 μg/ml kanamycin and 35 μg/ml chloramphenicol until OD_{600} reached 0.7–0.8. At this point, the culture was transferred to room temperature and induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 20–24 h of induction, cells were harvested by centrifugation and resuspended in 10 volumes of TN buffer (20 mM Tris-HCl, pH 8.0, 0.1 M NaCl) supplemented with 10 mM β-mercaptoethanol, 0.5% (w/v) Triton X-100, and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 20 μg/ml pepstatin A, 20 μg/ml leupeptin, and 1% (v/v) aprotinin). Cells were disrupted by sonication (5 times for 20 s with 1 min intervals for cooling on ice). Extracts were clarified by centrifugation at 50 000×*g* for 30 min at 4°C. Recombinant kinase was purified on a 5 ml bed volume of TALON resin (Clontech) following the manufacturer's instructions. Affinity purified kinase (approximately 5–10 mg of protein) was immediately supplemented with 5 mM dithiothreitol (DTT), desalted on a PD-10 column (Pharmacia) equilibrated in 25 mM Tris-HCl (pH 8.0) containing 0.5 mM EDTA, 5 mM DTT, 0.05% Triton X-100 (TEDT) plus the cocktail of protease inhibitors and loaded on MonoQ HR5/5 column (Pharmacia) equilibrated in TEDT at flow rate of 1 ml/min. The column was consecutively washed with 5 volumes of TEDT and developed with 20 volumes linear gradient of NaCl (from 0 to 500 mM) prepared in TEDT. The kinase containing fractions eluted at NaCl concentration of approximately 250–300 mM were pooled, made 50% (v/v) with glycerol and stored in small aliquots at −80°C.

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Abbreviations: PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; RPDK2, rat pyruvate dehydrogenase kinase, isoenzyme 2; E_1, pyruvate dehydrogenase component; E_2, dihydrolipoyl acetyltransferase component; E_3, dihydrolipoamide dehydrogenase; E_3BP, E_3-binding protein

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2.2. Expression and purification of the E_1-E_2 and E_1-E_2/E_3BP subcomplexes of human PDC

The sources of cDNAs for the components of human PDC and the construction of the respective bacterial expression vectors were reported previously [10]. Purification of E_1-E_2 and E_1-E_2/E_3BP subcomplexes was achieved by consecutive polyethyleneglycol-8000 (Sigma) fractionation, gel-filtration of Sepharose 4B (Pharmacia) and high-speed centrifugation. Some of the complexes were further purified by chromatography on Bio-Gel HT Gel (Bio-Rad) as described in [12]. Resulting preparations were stored at −80°C in small aliquots. Purified preparations of the recombinant human E_1 component were obtained as a generous gift from Dr. Mulchand S. Patel (Department of Biochemistry, SUNY at Buffalo). These preparations were further concentrated to the final protein concentration of 1 mg/ml using Centriplus concentrators (Amicon) and stored in small aliquots at −80°C.

2.3. PDK activity assay

Kinase activity was determined by following [^{32}P]phosphate incorporation from [γ-^{32}P]ATP into the $E_1\alpha$ subunit essentially as described previously for native PDC [12]. Phosphorylation reactions were set up in a final volume of 100 μl containing 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl, 1 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin (BSA), protein substrate, nucleotide substrate (the specific activity of [γ-^{32}P]ATP was approximately 200–500 cpm/pmol), and 5 μg/ml of the recombinant kinase at 37°C. The protein concentrations of the recombinant components of human PDC in the assay cocktail were as follows: the E_1 (0.5 mg/ml), the E_1-E_2 subcomplex (1.0 mg/ml), and the E_1-E_2/E_3BP subcomplex (1.0 mg/ml). For some experiments, E_1-E_2/E_3BP subcomplex was reconstituted with porcine heart E_3 component obtained from Sigma (0.1 mg of E_3 per 1.0 mg of E_1-E_2/E_3BP subcomplex) and used as a substrate for the recombinant RPDK2 at a final concentration of 1.0 mg/ml.

Phosphorylation reactions were initiated by addition of ATP after equilibration at 37°C for 30 s. Aliquots (20 μl) of the phosphorylation reaction were withdrawn after 20, 40, and 60 s of incubation and quenched by applying to dry discs of Whatman No. 3MM paper presoaked in 24% (w/v) trichloroacetic acid, 0.2 M phosphoric acid, 2 mM sodium pyrophosphate, and 1 mM ATP. Protein-bound radioactivity was determined as described previously [12]. A control (minus kinase) was included in each experiment. Kinase activities were calculated based on incorporation of [^{32}P]phosphate during the first 30 s of the reaction. All assays were conducted in triplicates. The rate of phosphorylation reaction was proportional to the amount of added kinase when RPDK2 was varied from 0.5–2.0 μg, suggesting that the protein substrate is not limiting under the conditions used. The concentrations of substrate (ATP) and effectors used in particular experiments are given in Section 3 and in the legends to the figures. Raw kinetic data were fitted and analyzed by using Grafit software (Eritacus Software). Shown are representative results obtained with one of the three preparations of RPDK2 that were analyzed for this study.

3. Results

3.1. The effects of E_2 or E_2/E_3BP subcomplexes on the enzymatic activity of RPDK2

PDK functions as an integral part of a multienzyme complex and its activity depends upon interaction with other components. Accordingly, several laboratories have demonstrated that the specific activity of native PDK substantially increases when kinase is bound to the complex [2], due presumably to tight binding with E_2/E_3BP-core [7]. In agreement with previous findings, the catalytic subunit of PDK showed very low catalytic efficiency using the recombinant E_1 component as a substrate, as would be expected for the native enzyme (Fig. 1). However, its enzymatic activity increased dramatically in the presence of the E_2 component, suggesting that, first, the catalytic subunit of the kinase is competent to respond to E_2-mediated activation without the involvement of the regulatory subunit and, second, that the transacetylase component alone is sufficient to support the enhanced catalytic function of the kinase. The E_3BP and E_3 components were found to be without any significant effect on kinase activity (Fig. 1). The E_3BP component had a rather slight inhibitory effect on kinase activity. These observations emphasize the previously mentioned importance of the transacetylase component of PDC toward maintaining enhanced kinase function [7]. They also show that the previously reported enhancement of kinase activity is due almost exclusively to the transacetylase component, in agreement with conclusions from Roche's laboratory [7]. The E_3BP component, which has considerable sequence homology to E_2 and was once implicated in the attachment of the kinase to the PDC core [13], appears to be without any substantial effect on the kinase activity, suggesting that E_2 is likely to be a primary site of kinase attachment.

3.2. Regulation of RPDK2 activity by NADH and NADH plus acetyl-CoA

The native kinase from several sources has been shown subject to regulation by NAD^+/NADH and CoA/acetyl-CoA ratios [14]. The recombinant catalytic subunit of PDK, like its native counterpart, is sensitive to the same type of regulation (Fig. 2). When holo-complex obtained by reconstitution of recombinant human E_1-E_2/E_3BP subcomplex with native porcine heart E_3 component was used as a substrate, kinase activity showed a characteristic increase in response to products of the dehydrogenase reaction – especially toward the simultaneous presence of NADH and acetyl-CoA. The transition from a mixture of NAD^+ and NADH with a molar ratio of 200:1 to a mixture with a molar ratio of 1:3 was associated with an approximately two-fold increase in kinase activity. The addition of acetyl-CoA to the former mixture of NAD^+/NADH produced further enhancement that reached almost five-fold when compared with kinase activity in the presence of NAD^+/NADH mixture at a molar ratio of 200:1 (Fig. 2, left panel). The stimulatory effects of NADH and NADH plus acetyl-CoA were apparent when the holo-PDC (E_1-E_2/E_3BP-E_3) was used as the substrate for the catalytic subunit. When the E_1-E_2/E_3BP subcomplex was used as a substrate, there was no apparent effect with neither NADH or

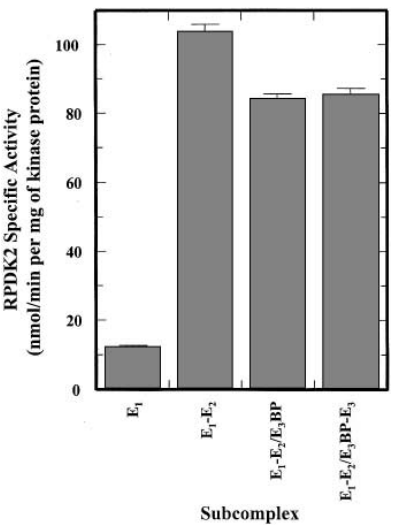

Fig. 1. The effects of E_2, E_2/E_3BP and E_2/E_3BP-E_3 subcomplexes of mammalian PDC on the specific activity of recombinant PDK2. The activity of RPDK2 was determined essentially as described in Section 2 using 100 μM ATP as a substrate. The activity with E_1 component alone was determined for 2 min of incubation.

NADH plus acetyl-CoA on kinase activity (Fig. 2, left panel). This observation is in agreement with the previous evidence suggesting that the effects of NADH and NADH plus acetyl-CoA reflect changes in the reduction and acetylated states of the lipoyl moieties of PDC [6] rather than direct allosteric effects of these compounds on the kinase molecule itself [15].

3.3. The effect of dichloroacetate on the activity of RPDK2

Dichloroacetate (DCA) is one of the most potent and specific synthetic inhibitors of native PDK. As shown by pioneering work from Philip Randle's laboratory [4], DCA mimics the effect of pyruvate, inhibiting kinase activity via a specific inhibitory allosteric site. Detailed kinetic analysis revealed that DCA is a non-linear hyperbolic inhibitor of kinase activity [5]. The mechanism of inhibition is quite complicated in that the sensitivity of the kinase to DCA is potentiated by ADP, which itself is a competitive inhibitor of kinase activity. Binding of the inhibitors is likely to occur in an ordered fashion, with the binding of ADP preceding the binding of DCA [5]:

$$E + ADP \rightleftharpoons E{\cdot}ADP + DCA \rightleftharpoons E{\cdot}ADP{\cdot}DCA.$$

This, in turn, makes it impossible to calculate the apparent K_i for DCA from inhibition studies in the absence of ADP. Therefore, in the present study the inhibition pattern of the recombinant catalytic subunit of PDK was analyzed at multiple concentrations of both inhibitors (DCA and ADP) and the respective kinetic data were analyzed by Dixon plot. DCA concentration was changed between 0–4.0 mM, and the ADP concentration between 0.1–0.8 mM. The concentrations of substrates were set at 100 μM for ATP and at 1 mg/ml for PDC. Under the described conditions, the inhibition pattern of the kinase activity by the two inhibitors was clearly synergistic (Fig. 3). When both DCA and ADP were present, the degree of inhibition was always greater than that observed at the same concentrations of DCA or ADP alone (data not shown). The apparent K_i value for DCA obtained from the secondary plot (Fig. 3, insert) was approximately 0.2 mM, which is almost identical to the apparent K_i value (0.21 mM) reported for bovine kidney enzyme [5]. In accord with observations by Pratt and Roche [5], the inhibition had a non-linear hyperbolic character.

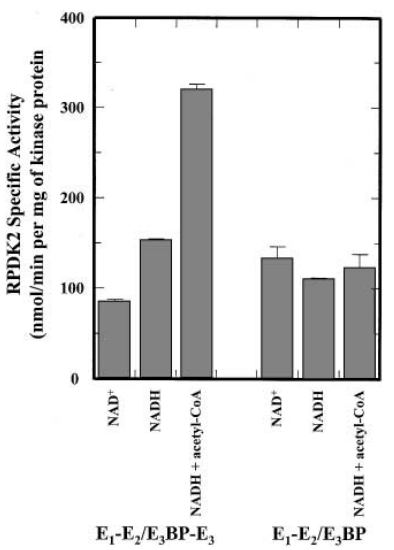

Fig. 2. The effect of E_3 component on the regulation of PDK2 activity by NADH and NADH plus acetyl-CoA. The kinase activity was determined as described in Section 2 with the addition of NADH and NAD^+ in a mixture to a final concentration of 0.6 and 0.2 mM respectively, and of acetyl-CoA to 50 μM. Effectors were added 30 s prior to ATP in order to allow for equilibration of the reactions catalyzed by E_2 and E_3 components. The final concentration of ATP was 100 μM. NAD^+ controls were made with the addition of a mixture of NAD^+ and NADH (molar ratio 200:1) to the final total concentration of 0.8 mM.

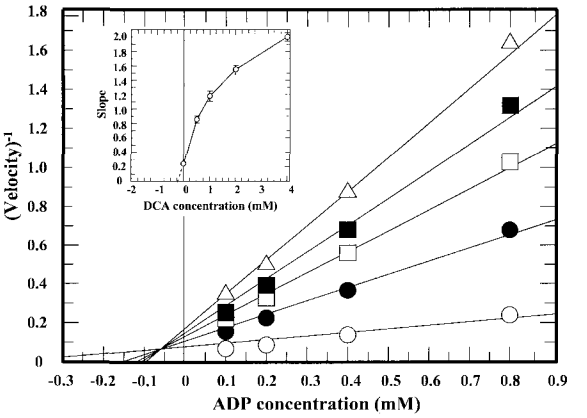

Fig. 3. Dixon plot of the effect of DCA on ADP inhibition of PDK2 activity. Kinase activity was determined without DCA (○) or in the presence of 0.5 mM (●), 1.0 mM (□), 2.0 mM (■), or 4.0 mM (△) DCA under conditions described in Section 3.

4. Discussion

When mammalian PDK was first purified from bovine kidney mitochondria [8] it appeared to be a heterodimeric enzyme consisting of a catalytic and a regulatory subunit. To date, the molecular role played by the regulatory subunit has not been defined, although it was suggested that it may serve as an adaptor that attaches the catalytic subunit to the core of the complex and/or mediates the effects of the products and substrates of the pyruvate dehydrogenase reaction on enzymatic activity of the catalytic subunit [8]. Further progress in characterization of the molecular role of the regulatory subunit has been slow because of the difficulties in purification of native PDK and resolution of its subunits. To complicate matters even further, later attempts by this [12] and Philip Randle's [16] laboratories to purify PDK from sources other than bovine kidney yielded kinase preparations which lacked the regulatory component. Surprisingly, these preparations appeared to be able to phosphorylate and inactivate PDC, and also to respond to effectors of kinase, suggesting that the catalytic subunit itself may be competent to carry out most of the kinase functions [12]. This conclusion should be applied with considerable caution, however, because of uncertainty with respect to the status of the regulatory subunit in the preparations of PDC used as substrate in those studies. Recent progress in the molecular cloning of cDNAs encoding for different components of mammalian PDC [11,12] have made PDK and its substrate PDC available as highly purified recombinant proteins. This has allowed a re-investigation of the regulation of the enzymatic activity of the catalytic subunit of PDK using recombinant proteins of well-defined subunit composition. As reported in the present manuscript, the recombinant PDK2, which is the most abundant isoenzyme of

PDK in kidney, appears to behave in a very similar, if not identical, manner to the native kinase from bovine kidney mitochondria. Its catalytic activity depends upon interaction with the E_2-core (Fig. 1) and is regulated by the products and substrates of the dehydrogenase reaction (Fig. 2), essentially as described previously for the kidney enzyme. The similarity of the recombinant and native kinase was quantitative as well as qualitative. For example, the magnitude of stimulation of kinase activity by the E_2 component, by NADH or by NADH plus acetyl-CoA was within the limits reported previously for the bovine kidney kinase [14]. Likewise DCA inhibited PDK2 activity synergistically with ADP, as reported previously for native kidney kinase [5].

Thus, the results reported here constitute strong evidence that the catalytic subunit of PDK is competent to respond to effectors of kinase activity, as well as to interact with the PDC core without assistance of the regulatory subunit. If one takes into account that we and others were unable to purify the kinase complexed with the regulatory subunit from heart [12] and liver [16] mitochondria, these results suggest that the regulatory subunit is not required for the kinase function, at least in some tissues. Indeed, the lack of evidence for a regulatory subunit in heart and liver raises the question of whether such a protein actually exists as an integral component of PDC. The fact that preparations of PDK appear to consist of several proteins with molecular weights ranging from 45 000 to 48 000 Da [8,12,13] may simply reflect the existence of multiple isoenzymes of PDK in different tissues [9]. On the other hand, the original evidence presented by Stepp et al. [8] for a regulatory subunit in bovine kidney is impossible to dismiss. It is interesting to note in this regard that the bovine kidney kinase binds to the complex extremely tightly. It remains bound to the E_2-core even during gel-filtration in the presence of solutions of high ionic strength [10]. Under comparable conditions the kinase from porcine heart, in contrast, appears to dissociate from E_2 almost completely [17]. These observations indicate that the regulatory subunit may be an adaptor that provides extremely strong binding of the kinase to the complex. This type of anchoring may be of importance for some as yet unidentified reasons in tissues like kidney, whereas in others, like heart or liver, the weaker direct binding of catalytic subunit to the E_2 component may be sufficient to elicit the regulatory features of the kinase.

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