FUNCTION OF THE NONIDENTICAL SUBUNITS OF MAMMALIAN PYRUVATE DEHYDROGENASE*

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Summary

The pyruvate dehydrogenase (PDH) component of the bovine kidney pyruvate dehydrogenase complex (PDC) contains two nonidentical subunits. PDH catalyzes the decarboxylation of pyruvate to produce α-hydroxyethylthiamine-PP (HETPP) and the reductive acetylation of the lipoyl moieties of dihydrolipoyl transacetylase with HETPP. Phosphorylation of PDH with PDH kinase and ATP markedly inhibits the first reaction but does not inhibit the second reaction. Since the α-subunit but not the β-subunit of PDH undergoes phosphorylation, these results suggest that the α-subunit catalyzes the first reaction and the β-subunit catalyzes the second reaction. Thiamine-PP reduces the rate of phosphorylation of PDC by PDH kinase and ATP. Phosphorylation of PDC increases the K_D of the PDC-Mg-thiamine-PP complex about 12-fold. It appears that the thiamine-PP binding site and the phosphorylation site on PDH influence each other and that HETPP is bound to PDH in a different orientation or possibly at a different site than is thiamine-PP.

The mammalian pyruvate dehydrogenase complex (PDC) catalyzes a coordinated sequence of reactions that can be represented by Reaction 1. This reaction is catalyzed by three enzymes, pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase, which act sequentially in that order (1). The pyruvate dehydrogenase (PDH) component of the complex catalyzes Reactions 2 and 3.

$$CH_3COCO_2H + CoA\text{-}SH + DPN^+ \longrightarrow CH_3CO\text{-}S\text{-}CoA + CO_2 + DPNH + H^+ \quad (1)$$

$$CH_3COCO_2H + \text{thiamine-PP} \longrightarrow CH_3CHOH\text{-thiamine-PP} + CO_2 \quad (2)$$

$$CH_3CHOH\text{-thiamine-PP} + [lipS_2] \longrightarrow [CH_3CO\text{-}S\text{-}lipSH] + \text{thiamine-PP} \quad (3)$$

PDH isolated from the bovine kidney and heart PDC possesses the subunit structure $\alpha_2\beta_2$ (2). The molecular weights of the α- and β-subunits are about 41,000 and 36,000, respectively. The α-subunit, but not the β-subunit, undergoes phosphorylation in the presence of PDH kinase and $MgATP^{2-}$ (2). This

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phosphorylation converts PDC to a form that is inactive in Reaction 1 (3). In this paper we report the effects of phosphorylation of PDH on its ability to catalyze Reactions 2 and 3 and on its ability to bind thiamine-PP.

MATERIALS AND METHODS

Highly purified preparations of bovine kidney PDC and its component enzymes were obtained as described previously (4).

Synthesis of 2-(α-hydroxyethyl)thiamine-PP (HETPP): HETPP was synthesized by a modification of the procedure of Ullrich and Mannschreck (5). The reaction mixture contained, per 1.5 ml, 10 μmoles of potassium pyruvate, 5 μmoles of thiamine-PP, 2 μmoles of $MgCl_2$, 30 μmoles of potassium phosphate buffer (pH 7.0), and 3 mg of purified kidney PDC. [2-^{14}C]Pyruvate was used to obtain ^{14}C-labeled HETPP. The mixture was incubated at 23° for 5 hours. Three volumes of boiling methanol were added, and the mixture was cooled and centrifuged. The supernatant fluid was concentrated in a vacuum evaporator and chromatographed on Whatman No. 3MM paper using n-propanol-1M ammonium formate (pH 5.0)-water (65:15:25) as described by Krampitz and Votaw (6). The band containing HETPP (R_f 0.41) was cut from the paper, the product was eluted with water, and the solution was lyophilized. The yield of HETPP was 5-15% (based on thiamine-PP). [α-^{14}C]HETPP contained equivalent amounts of ^{14}C-labeled hydroxyethyl moiety and thiamine-PP, as determined by the thiochrome method (7). Ferricyanide was added last, and the sample was read after 20 sec in a Farrand MK-1 spectrofluorometer (excitation at 370 nm, fluorescence emission at 440 nm).

Phosphorylation of PDH: The reaction mixture contained 4.56 mg of crystalline PDH (2), 0.22 mg of transacetylase-kinase subcomplex (2), 0.05 M phosphate buffer (pH 7.5), 2 mM dithiothreitol, 1 mM $MgCl_2$, 0.1 mM EDTA, and 4 mM NaF (to inhibit PDH phosphatase (4)) in a final volume of 1.3 ml. [γ-^{32}P]ATP (6.4 mCi/mmole) was added to give a concentration of 0.1 mM. The mixture was incubated at 23°, and 0.3-ml aliquots were withdrawn at 10, 30,

and 75 min and filtered immediately through a column of Sephadex G-25 (1 x 10 cm) which had been equilibrated at 4° with a solution containing 0.05 M phosphate buffer (pH 7.5), 0.1 mM dithiothreitol, 1 mM $MgCl_2$, and 10 mM NaF. Protein was determined by the biuret method (8), and protein-bound ^{32}P-labeled phosphoryl groups as described previously (4).

Reconstitution of PDC activity (Reaction 1): Samples (20 µg) of PDH or phosphorylated PDH and dihydrolipoyl transacetylase (63 µg) were incubated at 23° for 10 min in 0.2 ml of a solution containing 0.02 mM thiamine-PP, 0.1 mM DPN, 1 mM $MgCl_2$, 2 mM dithiothreitol, 10 mM NaF, and 0.05 M phosphate buffer (pH 7.5). Dihydrolipoyl dehydrogenase (40 µg) was added, and the incubation was continued for an additional 10 min. Aliquots (0.04-0.1 ml) were assayed for DPN-reduction activity as described previously (4).

$[^{14}C]CO_2$ evolution assay (Reaction 2): The reaction mixture contained 0.8 mM [1-^{14}C]pyruvate (0.6 mCi/mmole), 0.2 mM thiamine-PP, 1 mM dithiothreitol, 1 mM $MgCl_2$, 10 mM NaF, 0.04 M phosphate buffer (pH 7.0), and 20 µg of enzyme in a final volume of 0.5 ml. The mixture was incubated at 25° for 3 min. $[^{14}C]CO_2$ was determined as described by Schwartz and Reed (9). The values reported are corrected for $[^{14}C]CO_2$ produced in the absence of enzyme.

Reductive acetylation assay (Reactions 2 and 3): The reaction mixtures contained 0.03 mM [α-^{14}C]HETPP (4.8 mCi/mmole) or 0.13 mM [2-^{14}C]pyruvate (3 mCi/mmole) plus 0.1 mM thiamine-PP, 1 mM $MgCl_2$, 0.02 M phosphate buffer (pH 7.5), 10 mM NaF, 15 µg (Reactions 2 and 3) or 30 µg (Reaction 3) of PDH, and 170 µg of dihydrolipoyl transacetylase in a final volume of 0.25 ml. The mixtures were incubated at 4° or 25° for 1 min (Reactions 2 and 3) or 2 min (Reaction 3). Protein-bound ^{14}C-labeled acetyl groups were determined as described by Barrera *et al.* (2).

RESULTS AND DISCUSSION

Bovine kidney PDH catalyzes a reductive acetylation of the lipoyl moieties of dihydrolipoyl transacetylase (Reactions 2 and 3) in the presence

of [2-^{14}C]pyruvate, thiamine-PP and Mg^{2+} (2). PDH also catalyzes the evolution of [^{14}C]CO_2 from [1-^{14}C]pyruvate (Reaction 2). We have found that PDH can use [α-^{14}C]HETPP in place of pyruvate and thiamine-PP in the reductive acetylation reaction (Reaction 3). Kidney PDC can also use HETPP as substrate in the reduction of DPN at about 0.2% the rate obtained with pyruvate and thiamine-PP (Reaction 1). The rate-limiting step in the reaction with HETPP is apparently either binding of HETPP to PDH or removal of the α-proton of HETPP (5). An apparent K_m of about 0.025 mM was found for HETPP in the reduction of DPN. The ability to measure partial Reactions 2 and 3 permitted us to gain insight into the function of the α- and β-subunits of PDH.

The data presented in Table I show that phosphorylation of kidney PDC with PDH kinase and $MgATP^{2-}$ markedly inhibited Reaction 1 with pyruvate as substrate, but had little effect, if any, on the rate of this reaction with HETPP as substrate. This observation suggested that Reaction 2 was inhibited by phosphorylation of PDH. Direct evidence for this conclusion was obtained from studies with PDH and phosphorylated PDH. As shown in Table II, extensive phosphorylation of PDH with PDH kinase and Mg[γ-^{32}P]ATP^{2-} markedly inhibited its ability to catalyze Reaction 2, with [1-^{14}C]pyruvate as substrate, but did not inhibit its ability to catalyze Reaction 3, with [α-^{14}C]HETPP as substrate. The extent of inhibition paralleled the extent of phosphorylation of PDH. Inhibition of Reaction 2 by phosphorylation of PDH was also reflected in the inability of phosphorylated PDH to participate in overall Reaction 1, in the presence of dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase, and to catalyze the reductive acetylation reaction with [2-^{14}C]pyruvate as substrate (Reactions 2 and 3).

We have considered several possible functions for the two nonidentical subunits of PDH. One possibility is that the two subunits correspond to a catalytic and a regulatory subunit. Another possibility is that both subunits have a catalytic function. In our opinion, the simplest interpretation of the data is that the α-subunit, which undergoes phosphorylation, catalyzes Reaction

Table I

Effect of Phosphorylation on Enzymic Activity of PDC

Sample	Specific activity[a]	
	Pyruvate	HETPP
PDC	14.5	0.03
Phosphorylated PDC	0.3	0.03

[a]Micromoles DPNH formed/min/mg protein at 30°. The DPN-reduction assay (Reaction 1) was carried out as described previously (4). In the assays with pyruvate as substrate, the reaction was initiated by the addition of 5 μg of pyruvate dehydrogenase complex or 20 μg of the phosphorylated complex. In the assays with HETPP as substrate, pyruvate and thiamine-PP were omitted from the reaction mixture, 800 μg of enzyme complex was used, and the reaction was initiated by the addition of HETPP (final concentration, 0.075 mM).

Table II

Effect of Phosphorylation on Enzymic Activity of PDH[a]

Sample	^{32}P Content nmoles/mg protein	Reaction 1 μmoles/min	Reaction 2 nmoles/min	Reactions 2 + 3 nmoles/min		Reaction 3 nmoles/min
PDH	0	6.0	20.1	84.0	43.0	1.8
(^{32}P)PDH	1.6	4.7	15.5	70.8	35.0	2.1
(^{32}P)PDH	3.9	2.6	12.8	52.6	18.9	2.3
(^{32}P)PDH	8.2	0.7	6.3	18.0	2.6	2.4

[a]PDH was phosphorylated and assays were conducted as described in Materials and Methods. The values are expressed as rates/mg of PDH or (^{32}P)PDH. Reaction 1 was carried out at 30°, Reaction 2 and Reaction 3 at 25°, and Reactions 2 + 3 at 25° (column 4) or 4° (column 5). The substrates used in these reactions were: pyruvate (Reaction 1), [1-^{14}C]-pyruvate (Reaction 2), [2-^{14}C]pyruvate (Reactions 2 + 3), and [α-^{14}C]HETPP (Reaction 3).

2 and that the β-subunit catalyzes Reaction 3. Further testing of this proposal must await separation of PDH into its subunits under mild conditions (2).

Purified preparations of kidney PDC and the PDH component isolated from

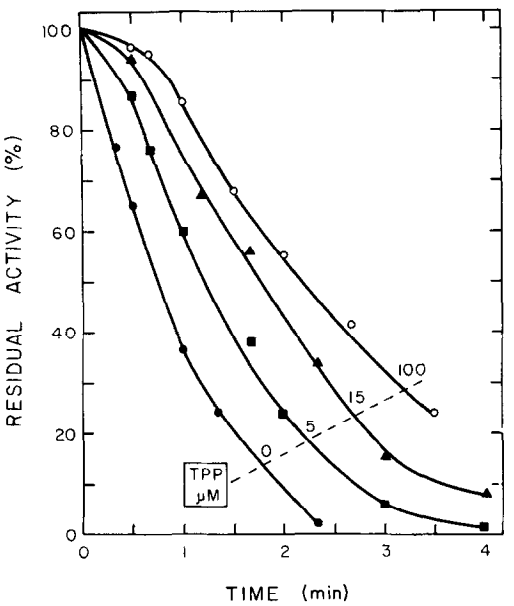

Fig. 1.

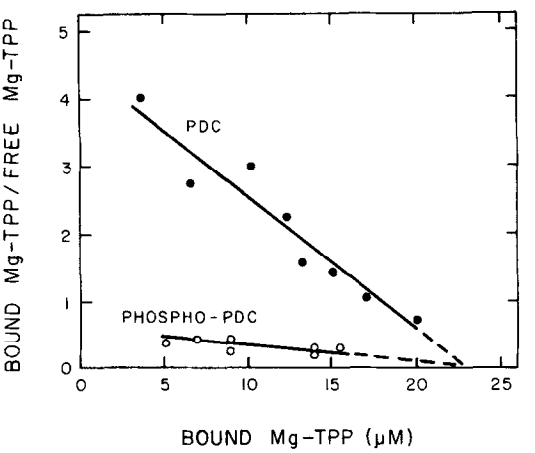

Fig. 2.

Fig. 1. Effect of thiamine-PP (TPP) on the rate of inactivation, i.e. phosphorylation, of PDC. The reaction mixtures contained 0.2 mg of bovine kidney PDC, 1 mM $MgCl_2$, 2 mM dithiothreitol, the indicated concentrations of thiamine-PP, and 0.1 mM ATP in a final volume of 0.2 ml of 0.05 M phosphate buffer (pH 7.5). PDC was preincubated with thiamine-PP for 2 min at 30°, and ATP was added last. Thiamine-PP did not affect the activity of the control, which lacked ATP. At the indicated times, aliquots were assayed for DPN-reduction activity (Reaction 1) as described previously (4).

Fig. 2. Determination of K_D (apparent) for the PDC-Mg-TPP and phosphorylated PDC-Mg-TPP complexes according to the method of Scatchard (11). The incubation mixtures contained 0.05 M phosphate buffer (pH 7.5), 5-100 μM thiamine-PP, 1 mM $MgCl_2$, 2 mM dithiothreitol, 10 mM NaF, and 0.64 mg of PDC or phosphorylated PDC (95% inactivated) in a total volume of 0.16 ml. The mixtures were incubated for 30 min at 23° and then transferred to a Metaloglass M129 ultrafiltration cell equipped with XM-50 Diaflo membranes. A nitrogen pressure of 30 psi was applied, and about 50 μl of ultrafiltrate was collected. Thiamine-PP was measured by the thiochrome method (7). The concentrations of Mg-thiamine-PP were calculated using a K_D of 0.407 mM (7).

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these preparations are deficient in thiamine-PP. We have observed that thiamine-PP reduces the rate of inactivation (i.e., phosphorylation) of PDC (Fig. 1) by PDH kinase and $MgATP^{2-}$. Since thiamine-PP does not affect the rate of phosphorylation of casein by the kinase and $MgATP^{2-}$ (10), its effect

is apparently exerted on PDH rather than on the kinase. We have also observed that phosphorylation of PDC decreases its ability to bind thiamine-PP. From the Scatchard plots (11) shown in Figure 2, apparent K_D values of about 5 μM for the PDC-Mg-thiamine-PP complex and about 60 μM for the phosphorylated PDC-Mg-thiamine-PP complex are obtained. These results suggest that the thiamine-PP binding site and the phosphorylation site on PDH influence each other. The various data also suggest that HETPP is bound to PDH in a different orientation or possibly at a different site than is thiamine-PP. The possibility should also be considered that binding of thiamine-PP to PDH may contribute to the regulation of the phosphorylation reaction (i.e., PDH kinase activity).

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