α-KETO ACID DEHYDROGENASE COMPLEXES, XI. COMPARATIVE STUDIES OF REGULATORY PROPERTIES OF THE PYRUVATE DEHYDROGENASE COMPLEXES FROM KIDNEY, HEART, AND LIVER MITOCHONDRIA*

BY TRACY C. LINN, FLORA H. PETTIT, FERDINAND HUCHO, AND LESTER J. REED

CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TEXAS (AUSTIN)

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Abstract.—The activity of the multienzyme pyruvate dehydrogenase complexes, isolated from mitochondria of beef kidney, beef heart, and pork liver, is regulated by phosphorylation and dephosphorylation. Phosphorylation and concomitant inactivation of each of the three complexes are catalyzed by an ATP-specific kinase, and dephosphorylation and concomitant reactivation are catalyzed by a phosphatase. The phosphatase has been separated from the other component enzymes of each pyruvate dehydrogenase complex, and the three phosphatases are functionally interchangeable. The kinase has been isolated from the beef kidney complex, and it is functional with the beef heart and pork liver complexes. ADP is competitive with ATP, and the ADP effect is more pronounced with the kidney kinase than with the liver and heart kinases. Pyruvate protects strongly the heart and liver pruvate dehydrogenase complexes and, to a lesser extent, the kidney complex against inactivation by ATP. Pyruvate apparently exerts its effect on the pyruvate dehydrogenase component of the complex, rather than on the kinase.

In a previous publication[1] we reported that the activity of the pyruvate dehydrogenase (PDH) complex from beef kidney mitochondria is subject to regulation by a phosphorylation-dephosphorylation reaction sequence. The site of this regulation is the pyruvate dehydrogenase component of the complex. Phosphorylation and concomitant inactivation of this component are catalyzed by an ATP-specific kinase (i.e., a PDH kinase), and dephosphorylation and concomitant reactivation are catalyzed by a phosphatase (i.e., a PDH phosphatase). The kinase is active at low levels of Mg^{++}, whereas the phosphatase requires a Mg^{++} concentration of about 10 mM for optimum activity.

This paper reports the results of comparative studies of the regulatory properties of the PDH complex isolated from mitochondria of beef kidney, beef heart, and pork liver. The activity of the PDH complex, from heart and liver, as well as from kidney, is subject to regulation by phosphorylation and dephosphorylation. The phosphorylation and dephosphorylation reactions are catalyzed, respectively, by a kinase and a phosphatase. Although the regulatory properties of the three complexes are qualitatively similar, there appear to be significant quantitative differences.

Materials and Methods.—Assay procedures and the sources of materials have been described previously.[1]

Purification of PDH complex: Mitochondria were isolated in 0.25 *M* sucrose and, after appropriate washing, were ruptured by freezing and thawing.[1, 2] Beef kidney mitochondria were washed once with 0.25 *M* sucrose and once with deionized water, and then they were resuspended in water (40 mg of protein/ml) prior to shell-freezing in Dry Ice-isopropanol. Pork liver mitochondria were washed three times with sucrose and once or twice with deionized water, and were resuspended in water prior to freezing. Beef heart mitochondria were washed once with sucrose and twice with 20 mM potassium phosphate buffer, pH 7.0, and were resuspended in the same buffer prior to freezing. The thawed suspensions were made 50 mM with respect to NaCl, the pH was adjusted to 6.5, and the suspensions were clarified by centrifugation. The PDH complex was purified by precipitation with protamine, ultracentrifugation, and isoelectric precipitation as described previously.[1] The initial protamine precipitation step was omitted in the case of the heart and liver complex. The specific activities (μmoles of DPNH formed/min/mg of protein at 25°) of the purified preparations of complex used in this investigation were: kidney, 7–10; heart, 5–10; and liver, 3–5.

Resolution of the PDH complex: The complex was separated into a dihydrolipoyl transacetylase fraction and a pyruvate dehydrogenase fraction containing a small amount of flavoprotein by gel filtration on Sepharose 4B at pH 9 as described previously.[1]

Isolation of kidney PDH kinase: Previous observations[1] indicated that kidney PDH kinase is a sulfhydryl enzyme. Data presented below reveal that the kinase is bound to the transacetylase component of kidney complex. Separation of the kinase and the transacetylase was accomplished by the following procedure. The transacetylase fraction obtained by resolution of beef kidney complex was dialyzed against 30 mM glycine, pH 8.7, to remove dithiothreitol. The dialyzed solution (30 mg of protein in 3.6 ml) was added, with gentle mixing, to a solution of 3.6 mg (10 μmoles) of *p*-hydroxymercuribenzoate in 0.4 ml of 0.32 *M* glycine, pH 8.7. The mixture was allowed to stand at room temperature for 30 min and then was applied to a column of Sephadex G-200 (bed volume, 60 ml), which had been equilibrated with a solution of 0.01 mM *p*-hydroxymercuribenzoate in 50 mM glycine, pH 8.7. The transacetylase peak emerged at an elution volume of 20 to 30 ml, and the kinase peak at an elution volume of 33 to 40 ml. The appropriate fractions were combined and adjusted to pH 7.2, and dithiothreitol was added to give a final concentration of 10 mM. The solutions were concentrated by membrane filtration.

Isolation of PDH phosphatase: PDH phosphatase was obtained at two stages of the purification of kidney PDH complex. The initial protamine precipitate,[1] which had first been discarded since it contained little (if any) complex, was found to be rich in phosphatase activity. An acetone powder of this precipitate was suspended in 20 ml of 0.1 *M* phosphate buffer, pH 7.5, containing 2 mM dithiothreitol. To this suspension were added 4 ml of 1% sodium ribonucleate, and the mixture was homogenized. The suspension was clarified by centrifugation and diluted with an equal volume of water containing 2 mM dithiothreitol, and the pH was adjusted to 5.2. The precipitate was dissolved in a solution containing 20 mM phosphate buffer, pH 7.5, 1 mM $MgCl_2$, and 2 mM dithiothreitol. The phosphatase activity was stable during storage of the protein for several weeks at 4°. Kidney PDH phosphatase activity was also present in the supernatant fluid remaining after ultracentrifugation of the complex.[1] The phosphatase was concentrated by precipitation at pH 5.2. This latter procedure was used to obtain PDH phosphatase from preparations of heart and liver complex.

Results.—Phosphorylation and dephosphorylation of PDH complex from liver and heart: Previous studies[1] showed that beef kidney complex is inactivated by incubation with low levels of ATP and that inactivation is accompanied by transfer to the complex of the terminal phosphoryl moiety of ATP. Similar results have been obtained with purified preparations of the complex from beef heart mitochondria and from pork liver mitochondria (Table 1). Essentially no radioactivity was incorporated into protein (in a trichloroacetic acid-precipitable form) from α-P^{32}-ATP, whereas a substantial amount of radioactivity was in-

TABLE 1. *Transfer of label from radioactive ATP to liver and heart PDH complex.*

ATP	Residual Enzymatic Activity (%)		Protein-bound Radioactivity (cpm/mg protein)	
	Liver complex	Heart complex	Liver complex	Heart complex
α-P^{32}	0	11	3,200	4,000
γ-P^{32}	0	11	291,000	508,000

The reaction mixtures contained 20 μmoles of phosphate buffer, pH 7.0; 1 μmole of $MgCl_2$; 2 μmoles of dithiothreitol; 0.01 μmole of α-P^{32}-ATP (150,000 cpm/mμmole) or γ-P^{32}-ATP (132,000 cpm/mμmole) in the case of liver PDH complex, and 0.02 μmole of α-P^{32}-ATP or γ-P^{32}-ATP in the case of heart complex; and 1.4 mg of heart complex or 0.7 mg of liver complex in a total volume of 1.0 ml. The mixtures were incubated at 30° for 30 min (liver complex) or 60 min (heart complex), and aliquots were assayed for protein-bound radioactivity and for their ability to oxidize pyruvate with DPN as electron acceptor.

corporated from γ-P^{32}-ATP. The preparations of phosphorylated and inactivated PDH complex were reactivated, with concomitant dephosphorylation, by incubation with 10 mM Mg^{++}. The data presented in Figure 1 illustrate the time course of the reciprocal changes in enzyme activity and protein-bound phosphoryl groups. The rate of inactivation of heart complex appeared to be considerably slower than that of liver and kidney PDH complex.[1] In contrast, heart complex appeared to undergo faster reactivation (and dephosphorylation) than did that of liver and kidney. As indicated below, the relatively slow rate of inactivation of heart PDH complex is due, at least in part, to a deficiency of PDH kinase.

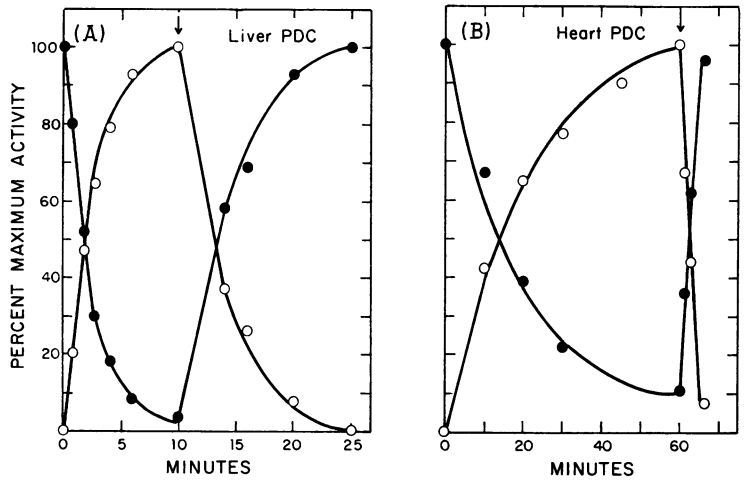

FIG. 1.—Time course of phosphorylation and dephosphorylation of liver and heart PDH complex. (*A*) The reaction mixture contained 20 μmoles of phosphate buffer, pH 7.3; 1 μmole of $MgCl_2$; 2 μmoles of dithiothreitol; 0.01 μmole of γ-P^{32}-ATP; and 1.0 mg of pork liver complex in a total volume of 1.0 ml. The mixture was incubated at 25°. At the indicated times, aliquots were assayed for DPN-reduction activity and for protein-bound radioactivity. At the time interval indicated by the vertical arrow, sufficient $MgCl_2$ was added to give a final concentration of 10 mM. To obtain a common ordinate, enzyme assays and protein-bound radioactivity have been expressed as percentage of maximum activity. (*B*) The reaction mixture contained 1.4 mg of beef heart complex and 0.02 μmole of γ-P^{32}-ATP. The temperature was 30°. Other components and conditions were as in (*A*).
●——●, DPN-reduction assay; O——O, protein-bound radioactivity. *PDC*, PDH complex.

Inactivation of liver and heart PDH complex with kidney PDH kinase: In a previous investigation,[1] kidney PDH complex was separated into a pyruvate dehydrogenase fraction and a transacetylase fraction by gel filtration on Sepharose 4B at pH 9. The former fraction underwent phosphorylation at a slow rate in the presence of ATP, and the rate was increased markedly in the presence of the transacetylase fraction. These results did not permit an unequivocal decision as to whether the kinase is associated with the pyruvate dehydrogenase component or with the transacetylase component of kidney PDH complex. The observation that beef heart complex (and some preparations of the pork liver complex) underwent relatively slow inactivation in the presence of ATP—suggesting that these preparations were deficient in kinase—provided an opportunity to settle this problem. Accordingly, the pyruvate dehydrogenase and transacetylase fractions obtained by resolution of kidney PDH complex were incubated separately for a short period (5 min) with preparations of liver and heart complex in the presence of ATP, and the extent of inactivation of these preparations was determined. The pyruvate dehydrogenase fraction from kidney complex exhibited only slight kinase activity with the liver and heart, complex, whereas the kidney transacetylase fraction was very active (Table 2).

TABLE 2. *Inactivation of liver and heart PDH complex with kidney PDH kinase.*

PDH complex	Fraction added	Decrease in DPN-reduction activity (%)
Kidney	None	79
Liver	None	8
Liver	LTA	82
Heart	None	14
Heart	PDH	17
Heart	LTA	87
Heart	Kinase	71

The reaction mixtures contained 20 μmoles of phosphate buffer, pH 7.3; 1 μmole of $MgCl_2$; 2 μmoles of dithiothreitol; 0.5 mg of PDH complex; and, where indicated, 0.2 mg of kidney transacetylase (LTA) fraction, 0.3 mg of kidney PDH fraction, or 0.1 mg of kidney kinase fraction in a total volume of 1.0 ml. The latter three fractions were obtained as described in *Materials and Methods*. Aliquots (0.02 ml) were taken for assay of DPN-reduction activity. ATP (0.01 μmole) was added to each mixture, the mixtures were incubated at 25° for 5 min, and 0.02-ml aliquots were reassayed for DPN-reduction activity.

These data indicate that the kinase is associated with the transacetylase component of kidney PDH complex and that the kidney kinase is functional with both liver and heart complexes. In subsequent experiments, the kidney kinase was separated from the kidney transacetylase (see *Materials and Methods*), and the isolated kinase was shown to be effective in inactivating the heart complex (Table 2).

Functional identity of PDH phosphatases from kidney, liver, and heart: Previous studies[1] indicated that PDH phosphatase is less strongly bound to kidney PDH complex than is PDH kinase, with the result that variable amounts of the phosphatase are released during the purification of the kidney complex. The phosphatase that remained bound to the PDH complex was largely removed by isoelectric precipitation of the complex, followed by ultracentrifugation of the dissolved precipitate. Similar observations have been made with the prepara-

tions from heart and liver. The availability of preparations of kidney, heart, and liver complex that were deficient in phosphatase, and preparations of the corresponding phosphatases as well, permitted testing of the interchangeability of the phosphatases. The PDH complex preparations were inactivated by incubation with a minimum amount of ATP. The inactivated (and phosphorylated) preparations were incubated with 10 mM Mg^{++} in the presence and absence of PDH phosphatase preparations from kidney, liver, or heart, and the extent of reactivation of the preparations of PDH complex was determined. Typical data are presented in Table 3. These results indicate that the PDH phosphatases from kidney, liver, and heart mitochondria are functionally identical.

TABLE 3. *Functional identity of PDH phosphatases from kidney, liver, and heart.*

Inactivated PDH complex	Phosphatase added	Activity regained (%)
Kidney	None	13
Kidney	Kidney	87
Kidney	Liver	80
Kidney	Heart	96
Liver	None	0
Liver	Kidney	78

The reaction mixtures contained 20 μmoles of phosphate buffer, pH 7.3; 1 μmole of $MgCl_2$; 2 μmoles of dithiothreitol; 0.01 μmole of ATP; 0.5 mg of PDH complex; and, where indicated, 0.1, 0.3, and 0.2 mg, respectively, of kidney, liver, or heart PDH phosphatase in a total volume of 1.0 ml. The phosphatase preparations were obtained as described in *Materials and Methods*. The mixtures were incubated at 25° until at least 85% of the DPN-reduction activity had disappeared. Then, sufficient $MgCl_2$ was added to each mixture to give a final concentration of 10 mM. Incubation was continued for 5 min and aliquots (0.02 ml) were reassayed for DPN-reduction activity.

Inhibition of PDH kinase by ADP: ADP inhibited inactivation of kidney, liver, and heart PDH complex by ATP (Fig. 2), and this inhibition was competitive with respect to ATP. AMP, GDP, adenosine 3′,5′-phosphate, and acetyl CoA had little effect, if any. Control experiments showed that ADP prevented incorporation into the complex of P^{32}-labeled phosphoryl groups from γ-P^{32}-ATP. Although beef kidney and pork liver PDH complex appeared to be inactivated at lower levels of ATP than that of beef heart,[3] it was not possible to obtain valid K_m values for ATP from the data presented in Figure 2. It should be noted that ATP reacts stoichiometrically and irreversibly with PDH complex, and the extent of inactivation of the complex at a given concentration of ATP is a function of the kinase concentration and the incubation period. Under conditions where a valid assay for PDH kinase activity obtains, the apparent K_m value for ATP was found to be about 0.02 mM for heart complex and about 0.09 mM for that of kidney. The apparent K_i values for ADP were about 0.11 and 0.08 mM, respectively. Details of these experiments will be presented in a subsequent publication.

Effect of pyruvate on inactivation of kidney, liver, and heart PDH complex by ATP: Pyruvate protected kidney, liver, and heart PDH complex against inactivation by ATP (Fig. 2). The apparent K_m values for pyruvate are about 0.044 mM for kidney complex and 0.035 mM for that of heart. Under the assay conditions used, 0.5 mM pyruvate provided almost complete protection of

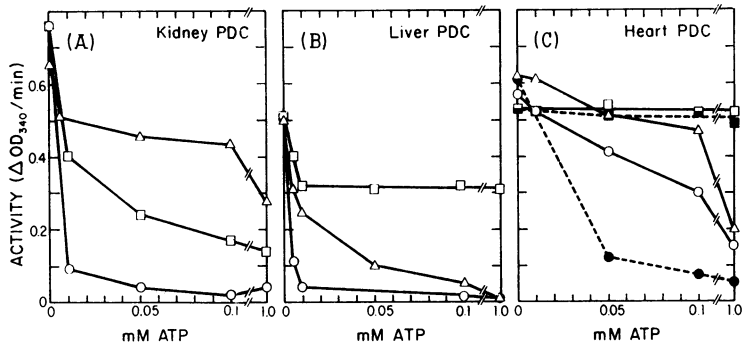

FIG. 2.—Effects of ADP and pyruvate on inactivation of kidney, liver, and heart PDH complex by ATP. (*A*) Reaction mixtures contained 4 μmoles of phosphate buffer, pH 7.3; 0.2 μmole of $MgCl_2$; 0.4 μmole of dithiothreitol; 0.14 mg of kidney complex; 0.2 μmole of ADP or 0.1 μmole of pyruvate, where indicated; and the indicated concentrations of ATP in a total volume of 0.2 ml. The ATP was added last. The mixtures were incubated at 30° for 6 min, and 0.02-ml aliquots were taken for assay of DPN-reduction activity. (*B*) Reaction mixtures contained 0.16 mg of pork liver complex. (*C*) Reaction mixtures contained 0.17 mg of beef heart complex; 0.02 μmole of $MgCl_2$; and, where indicated, 0.02 mg of kidney transacetylase fraction (as a source of kinase). The mixtures were incubated at 30° for 10 min. Other components and conditions were as in (*A*).

○——○, No ADP or pyruvate; △——△, 1 mM ADP; □——□, 0.5 mM pyruvate; ●- - -●, kidney kinase added, but no ADP or pyruvate; ■——■, kidney kinase and 0.5 mM pyruvate added.

heart PDH complex over a 200-fold range of ATP concentration (0.005–1.0 mM). The extent of protection of liver and kidney complex by pyruvate was about 60 and 20 per cent, respectively. α-Ketobutyrate, which also serves as a substrate for PDH complex of heart, kidney (apparent K_m = 0.12 mM), and liver, was about 80 per cent as effective (at 0.5 mM) as pyruvate in protecting heart complex against inactivation by ATP. α-Ketoisovalerate was not oxidized by heart PDH complex (with DPN as electron acceptor), nor did it protect the latter complex against inactivation by ATP. α-Ketoglutarate and oxaloacetate were also ineffective.

In the presence of kidney kinase, heart PDH complex was inactivated at substantially lower levels of ATP (Fig. 2*C*), and the rate of inactivation was similar to that observed with kidney complex. Nevertheless, the protective effect of pyruvate against inactivation of the heart complex by ATP remained essentially the same. These data are interpreted as indicating that pyruvate exerts its effect on the pyruvate dehydrogenase component of the complex, rather than on the kinase.

Discussion.—The data reported in this communication indicate that the activity of the pyruvate dehydrogenase complex from pork liver and beef heart mitochondria, as well as from beef kidney mitochondria, is subject to regulation by phosphorylation and dephosphorylation. A preliminary investigation[4] has revealed that the activity of partially purified preparations of PDH complex from beef liver mitochondria is also regulated by this control mechanism. Phosphorylation and concomitant inactivation of each complex are catalyzed by an ATP-specific kinase, and dephosphorylation and concomitant reactivation are

catalyzed by a phosphatase. The site of this regulation has been shown,[1] in the case of beef kidney complex, to be the pyruvate dehydrogenase component of the complex. Presumably, the pyruvate dehydrogenase component of beef heart and pork liver PDH complex also undergoes phosphorylation and dephosphorylation. The PDH phosphatases from kidney, heart, and liver are functionally interchangeable, and the kidney PDH kinase is functional with both the heart and liver complex. It appears that the concentration of Mg^{++} plays an important role in regulation of the phosphorylation-dephosphorylation reaction sequence. The kinase is active at low levels of Mg^{++}, whereas the phosphatase requires a Mg^{++} concentration of about 10 mM for optimum activity.

Although the regulatory properties of PDH complex from kidney, liver, and heart are qualitatively similar, there appear to be significant quantitative differences. ADP is competitive with ATP, and this effect is more pronounced with the kidney kinase than with the liver and heart kinases. On the other hand, pyruvate exerts a pronounced protective effect on heart and liver complex and a lesser protective effect on that of kidney. When kidney kinase was added to the heart complex, the response of the system to ATP resembled that observed with kidney complex. Nevertheless, kidney kinase did not alter the extent of protection of heart complex by pyruvate. α-Keto acids which serve as substrates for heart complex (pyruvate and α-ketobutyrate) are effective in protecting this complex against inactivation by ATP, whereas nonsubstrate α-keto acids (α-ketoisovalerate and α-ketoglutarate) are ineffective. These observations indicate that pyruvate acts on the pyruvate dehydrogenase component of PDH complex, protecting this component against phosphorylation and concomitant inactivation by the kinase and ATP. Since the apparent K_m values for pyruvate are approximately the same for kidney and heart pyruvate dehydrogenase, and yet the pyruvate effect is considerably more pronounced with heart pyruvate dehydrogenase than with kidney pyruvate dehydrogenase, it appears that pyruvate exerts its effect at a site on pyruvate dehydrogenase other than its catalytic center.

In a previous publication[1] it was surmised that the kinase is a regulatory subunit of the pyruvate dehydrogenase component of the beef kidney PDH complex. The present investigation demonstrates that such is not the case, but rather that the kinase is bound to the transacetylase component of the complex. Pyruvate dehydrogenase and dihydrolipoyl dehydrogenase (flavoprotein) are also bound to the transacetylase.[1, 2] The transacetylase apparently orients these three enzymes in a specific manner, thereby facilitating interactions between the active sites of these enzymes. Although it remains to be determined whether or not the PDH phosphatase is also bound to the transacetylase, it has been observed[1] that the transacetylase facilitates dephosphorylation of phosphorylated pyruvate dehydrogenase by the phosphatase. Procedures have been developed for separation and partial purification of the kidney kinase and the kidney, heart, and liver phosphatases, thus opening the way for further characterization of these enzymes.

The significance of the regulation of the activity of the PDH complex by phosphorylation and dephosphorylation with respect to control of the direction of

pyruvate metabolism in liver and kidney mitochondria, i.e., whether pyruvate is oxidized to acetyl CoA or carboxylated to oxaloacetate, has been discussed elsewhere.[1, 5] We are not unaware of other possible physiological implications of these findings, e.g., with respect to organ function. It has been reported[6] that the activity of pig heart PDH complex is inhibited by the products of pyruvate oxidation, acetyl CoA and DPNH, and that these inhibitions are reversed by CoA and DPN, respectively. The relative importance of this type of regulation versus the phosphorylation-dephosphorylation mechanism remains to be determined.

Note added in proof: After this manuscript was submitted a brief communication by O. Wieland, and B. v. Jagow-Westermann (*FEBS Letters*, **3**, 271 (1969)) appeared, which described similar observations on the regulatory properties of PDH complex from pig heart muscle.

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[1] Linn, T. C., F. H. Pettit, and L. J. Reed, these PROCEEDINGS, **62**, 234 (1969).

[2] Ishikawa, E., R. M. Oliver, and L. J. Reed, these PROCEEDINGS, **56**, 534 (1966).

[3] The purified preparations of PDH complex from beef heart exhibited little, if any, ATPase activity.

[4] Linn, T. C., and L. J. Reed, unpublished data.

[5] Reed, L. J., in *Current Topics in Cellular Regulation*, ed. B. L. Horecker and E. R. Stadtman (New York: Academic Press, in press), vol. 1.

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