

Interconversion of Phospho- and Dephospho- Forms of Pig Heart Pyruvate Dehydrogenase*

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Abstract. Pyruvate dehydrogenase from pig heart exists in active and inactive forms. Interconversion from the active (dephospho) form into the inactive (phospho) form is catalyzed by an ATP-dependent kinase. Conversely the enzyme is reactivated by a phosphatase which removes the phosphate group from the protein. By gradient centrifugation pyruvate dehydrogenase was prepared free of phosphatase but still containing the kinase. Reactivation of pyruvate dehydrogenase is stimulated by adenosine 3',5'-cyclic phosphate. There is incorporation of ^{32}P from $\gamma\text{-}^{32}\text{P}\text{-ATP}$ into the protein fraction containing the phosphatase and this phosphorylation reaction is also stimulated by adenosine 3',5'-cyclic phosphate. The participation of this phosphate in the pyruvate dehydrogenase interconversion system suggests that, in heart muscle, pyruvate oxidation may be under hormonal control by a mechanism similar to that involved in the regulation of glycogen synthesis and breakdown.

We have reported^{1, 2} that pyruvate dehydrogenase (PDH) (EC 1.2.4.1) from pig heart exists in active and inactive forms similar as described for the beef kidney enzyme.³ It was suggested that conversion of an active to the inactive form occurs by way of an ATP-dependent phosphorylation reaction (kinase reaction), and that the reverse reaction is catalyzed by a phosphatase. We now report the preparation of PDH free of phosphatase but still containing the kinase. Furthermore, we could show that addition of adenosine 3',5'-cyclic phosphate (3',5'-cyclic phosphate) *in vitro* stimulates reactivation of the phosphoenzyme.⁴

Materials and Methods. Coenzymes and other reagents were from the same sources and of the same purity as those used in earlier studies.^{1, 5} The magnesium content of commercial ATP preparations was lowered by one recrystallization from ethanol-water-EDTA. The 3',5'-cyclic nucleotides were a gift of Boehringer and Sons. $\gamma\text{-}^{32}\text{P}\text{-ATP}$ was purchased from the Radiochemical Centre, Amersham, England. Incorporation of ^{32}P from $\gamma\text{-}^{32}\text{P}\text{-ATP}$ into protein was measured according to Mans and Novelli.⁶ Filter paper disks no. 595 with a diameter of 2 cm from Schleicher and Schüll were used.

Purification of PDH: For the isolation and purification of pig heart pyruvate dehydrogenase the procedure of Scriba and Holzer⁷ was followed up to the step where the enzyme remains in the supernatant fluid after centrifugation at $22,000 \times g$. The $22,000 \times g$ supernatant solution was then dialyzed overnight against two changes of a large volume of 20 mM potassium phosphate buffer, pH 7.0. The small amount of precipitate formed during dialysis was not removed and the pH of the solution was brought to 6.1 by dropwise addition of 1% acetic acid. A precipitate was removed by

centrifugation at $10,000 \times g$ for 5 min. The clear supernatant fluid was brought to pH 5.6 with 1% acetic acid. The yellow precipitate that contained up to 60% of the PDH activity in the dialyzed $22,000 \times g$ supernatant solution was collected by a centrifugation at $10,000 \times g$ for 5 min. The precipitate was dissolved in a small volume of 20 mM potassium phosphate, 2.5 mM 2-mercaptoethanol, and 2 mM $MgCl_2$ buffer solution, pH 7.0. Addition of solid potassium bicarbonate aids in solubilizing the pH 5.6 precipitate. Care must be taken that the final pH does not exceed a value of 7.2 because the enzyme is inactivated at alkaline pH. The clear enzyme solution (1.5 ml \approx mg protein/ml) was layered on top of a linear glycerol gradient from 10 to 50% glycerol. The enzyme preparation was spun for 7 hr at 25,000 rpm in the SW-25-1 rotor of the Spinco model L2. Portions of 1 ml were collected for determination of enzyme activity and protein concentrations.⁸ Up to 40% glycerol did not interfere with the Lowry procedure for protein determination. All steps of the enzyme purification were performed at 4°C.

Assay of pyruvate dehydrogenase activity: Pyruvate dehydrogenase activity was determined spectrophotometrically by recording NAD reduction either with lactate or pyruvate as substrate. Activities are expressed as μ moles NADH formed per minute at 25°C under the conditions described.⁵

Results. Purity of PDH complex: The purification scheme starting with the $22,000 \times g$ supernatant step is given in Table 1. The maximal specific activity listed in Table 1 is two to three times lower than the maximal specific activity of the enzyme after preincubation with Mg^{++} and the phosphatase. This suggests that only 30–50 per cent of the purified enzyme are present in the active, i.e., dephosphoform. In our purified PDH preparation, no adenylate kinase or lactate dehydrogenase activity was found. α -Ketoglutarate dehydrogenase activity was no more than 5 per cent of that of the PDH activity. Malate dehydrogenase and creatine phosphokinase activities were about 1 per cent of that of the purified PDH activity. On acrylamide gel electrophoresis only a very small amount of protein traveled ahead of the band containing the PDH. The latter did not move from the start. An electron micrograph of the purified PDH complex is shown in Figure 1.

Pig heart PDH was found to be labile at alkaline pH. At pH 8.2 and 7.8, enzyme activity decreased within one hour at 27°C by 96 and 67 per cent, respectively. Similar observations were made by Hayakawa *et al.*¹⁰ No loss of activity occurred at these pH values at 0°C. On the other hand, addition of Mg^{++} or glutathione could not prevent inactivation. At neutral pH and in frozen state, PDH was stable for several weeks. The inactive phosphoenzyme was likewise stable under these conditions. It could be fully reactivated.

TABLE 1. *Purification of PDH from pig heart muscle.*

	Protein (mg)	Activity (U)	Spec. act. (U/mg)	Yield (%)
22,000 $\times g$ supernatant (dialyzed)	1012	146	0.14	100
Isoelectric precipitation				
Supernatant, pH 6.1	410	65.7	0.16	45
Precipitate, pH 5.6	86	60	0.70	41
Gradient centrifugation (fractions spec. act. ≥ 1.8 combined)	27	58.5	2.16	40

Enzyme activities refer to the assay system with pyruvate as substrate. Where the assay was performed with lactate, the appropriate correction (factor approx. 3) has been applied.

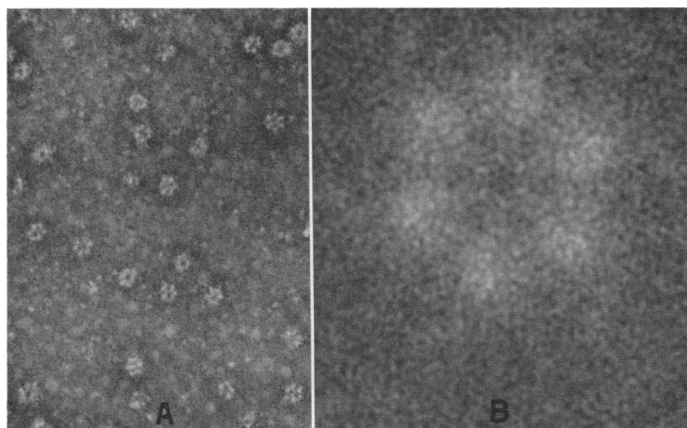


FIG. 1.—Electron micrographs of the PDH-complex from pig heart muscle. (A) A droplet from a gradient fraction (0.4 mg protein/ml, spec. act. 3.6) was diluted about five times by mixing with a drop of neutralized 2% phosphotungstic acid (precooled to 4°C). Collodium-carbon-coated copper grids were put on the protein-stain mixture for a few seconds and excess material was then removed with lens paper. After air drying the specimens were examined in an AEI-EM 6 B electron microscope, equipped with an anticontamination device at 80 kv. Electron optical magnification $\times 80,000$, final magnification $\times 120,000$. The electron micrographs of the PDH-complex shown here and those published by Hayakawa *et al.*¹⁰ look alike. (B) Picture of a single PDH-complex according to the technique of Markham *et al.*⁹ Rotational symmetry ($n = 6$) in the multienzyme complex is obvious. Final magnification $\times 1.5 \times 10^6$. We are much indebted to Prof. F. Miller, the University of Munich, for allowing us to perform the electron microscopic studies in his institute and for much useful advice.

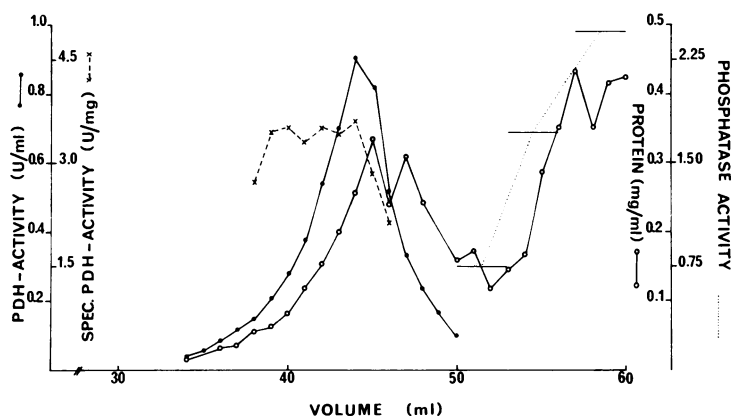


FIG. 2.—Separation of phospho-PDH-phosphatase and the PDH complex by gradient centrifugation. The procedure is described in *Materials and Methods*. In the experiment shown, time of centrifugation was 5 hr. The horizontal bars indicate phosphatase activity measurements on the pooled gradient fractions. The rate of reactivation of phospho-PDH was used as measure of phosphatase activity. Incubation was for 30 min at 25°C.

Removal of phospho-PDH phosphatase from the PDH complex: Purified PDH after gradient centrifugation still contained the kinase but was free of phosphatase activity. This is indicated by complete inactivation with ATP, whereas reactivation was dependent on addition of a second protein fraction present in the upper layer of the gradient (see Fig. 2). Increasing the concentration of Mg^{++} alone did not lead to reactivation.

Phosphorylation of PDH: Incorporation of ^{32}P into the enzyme protein after incubation with $\gamma\text{-}^{32}P\text{-ATP}$ made it seem possible that inactivation and reactivation of heart muscle PDH might be related to phosphorylation and dephosphorylation of the enzyme. As shown in Figure 3 there actually is an inverse relationship between inactivation and ^{32}P incorporation, and vice versa. Moreover, reactivated PDH has a much higher activity as did the enzyme at the

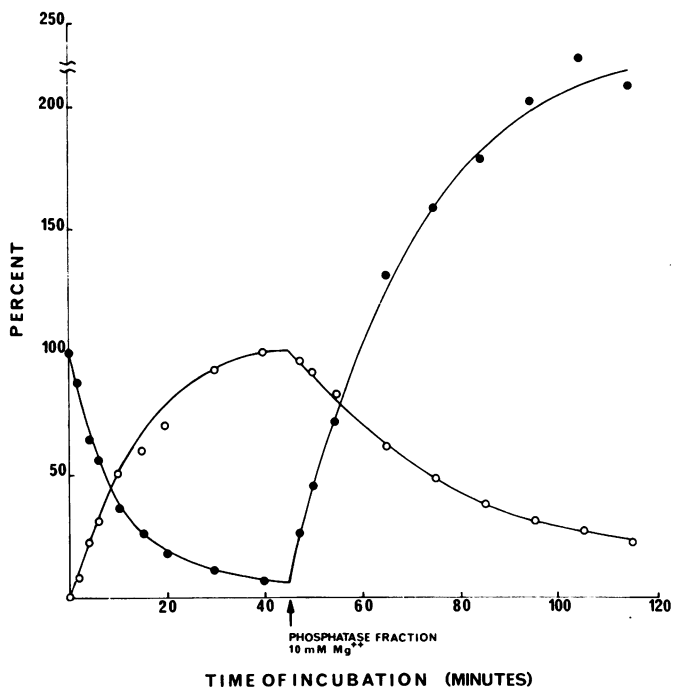
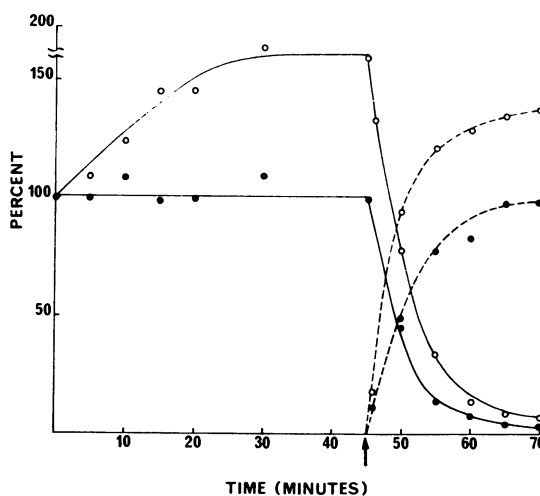


FIG. 3.— ^{32}P -incorporation and release from the pig heart muscle PDH-complex in the course of enzyme inactivation and reactivation. The reactivation mixture contained in 500 μ l of 20 mM potassium phosphate buffer, pH 7.0, 530 μ g of purified PDH, 18 μ M $\gamma\text{-}^{32}P\text{-ATP}$ (9.7×10^6 cpm), 1.9 mM $MgCl_2$. Incubation was at 25°C. Two samples, 10 μ l each, were taken at the times indicated for determination of PDH activity and for measurement of protein-bound radioactivity. At the time indicated by arrow, 20 μ l phosphatase solution (214 μ g) and 10 μ l of a 208 mM $MgCl_2$ solution were added to the remaining 220 μ l of the incubation mixture. The results are expressed as percentage of the original enzyme activity (at 0 time) or as percentage of the maximal protein-bound radioactivity, respectively. 100% PDH activity represents 0.28 units and 100% radioactivity represents 113,000 cpm. The increase in PDH activity after reactivation was discussed (see text). ●—●, enzyme activity. ○—○, protein-bound radioactivity.

start of the inactivation-activation cycle. This might be explained by assuming that initially the purified enzyme is present as a mixture of active and inactive forms. This suggestion is supported by the results shown in Figure 4. As may be seen, preincubation of PDH with the phosphatase preparation and Mg^{++} results in a marked increase in enzyme activity when compared with the control incubated with Mg^{++} alone. After addition of γ - ^{32}P -ATP, both the dephosphorylated enzyme and the enzyme preparation not treated with the phosphatase are inactivated to the same extent. But as one might expect a much higher amount of ^{32}P was incorporated into the dephosphorylated enzyme, i.e., the enzyme pretreated with phosphatase prior to addition of γ - ^{32}P -ATP.

FIG. 4.— ^{32}P -incorporation and release from activated pig heart muscle PDH. 90 μ l of purified PDH (332 μ g protein) were incubated at pH 7.0 with 30 μ l of a 25 mM $MgCl_2$ solution and 30 μ l (155 μ g protein) of the phosphatase preparation (O—O), or 30 μ l of a 20 mM potassium phosphate solution, pH 7.0, in place of the phosphatase preparation (●—●). Incubations were for 45 min at 25°C. At the time indicated by arrow, 40 μ l of a 25 μ M solution of γ - ^{32}P -ATP (1.8×10^6 cpm), 10 μ l of a 13.5 mM solution of EDTA, 10 μ l of a 20 mM potassium phosphate solution, pH 7.0, and 15 μ l of the phosphatase preparation (77.5 μ g) (●---●), or 15 μ l of a 20 mM potassium phosphate solution, pH 7.0, in place of the phosphatase preparation (O---O) were added. Enzyme activity (—) and radioactivity (---) were measured as described in the legend to Fig. 3.



The effect of 3',5'-cyclic AMP on reactivation of PDH: Addition of 3',5'-cyclic AMP markedly enhances reactivation of ATP-inactivated preparations (Fig. 5). Once ATP is removed from the ATP-inactivated PDH by dialysis, 3',5'-cyclic AMP no longer has an effect. Thus ATP is required for the cyclic nucleotide effect. Other cyclic nucleotides, i.e., 3',5'-CMP, 3',5'-GMP, 3',5'-IMP, 3',5'-UMP, and also 5'-AMP were ineffectual. Moreover, cyclic AMP had no effect on PDH-kinase. If one assumes that the phosphatase likewise exists as phospho- and dephosphoenzyme, then it would be conceivable that cyclic AMP acts on the phosphorylation of the phosphatase. In order to gain information on that point the following experiments were performed.

Incorporation of ^{32}P from γ - ^{32}P -ATP into the phosphatase: The phosphatase preparation was incubated with γ - ^{32}P -ATP. As shown in Figure 6, ^{32}P is incorporated into the phosphatase fraction also and this phosphorylation reaction is highly dependent upon 3',5'-cyclic AMP. Without cyclic AMP very little

^{32}P is incorporated into the protein. Addition of saturating concentrations of cyclic AMP increased ^{32}P -incorporation about tenfold. From these data one estimates a K_A -value for 3',5'-cyclic AMP of $2 \times 10^{-7} \text{ M}$. Aminophylline further enhanced the cyclic AMP effect. Without aminophylline 100 times higher nucleotide concentrations are needed to produce the same effect.

Temperature dependency of ^{32}P -incorporation into phosphatase: The possibility that ^{32}P is merely adsorbed onto the phosphatase is made unlikely by

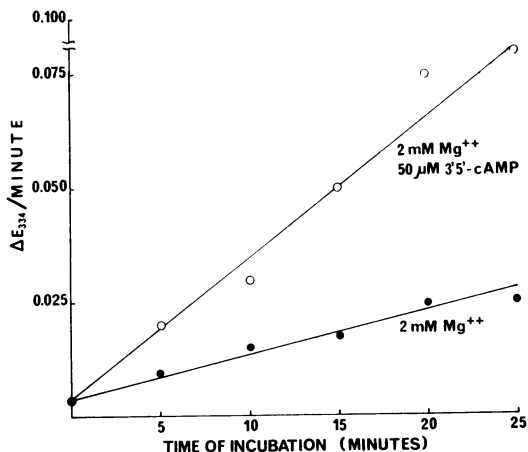


FIG. 5.—Effect of 3',5'-cyclic AMP on reactivation of phospho-PDH. Purified PDH was inactivated, essentially as described in the legend to Fig. 3, with the exception that unlabeled ATP was used. Phospho-PDH (40 μl corresponding to 11 μg protein), 10 μl of a 14 mM MgCl_2 solution, 10 μl of a 20 mM potassium phosphate solution, pH 7.0, 5 μl of the phosphatase preparation (57 μg protein) and 5 μl of a 0.7 mM solution of 3',5'-cyclic AMP or as controls 5 μl H_2O were incubated at 25°C. Aliquots (10 μl) were assayed for PDH activity, as described.

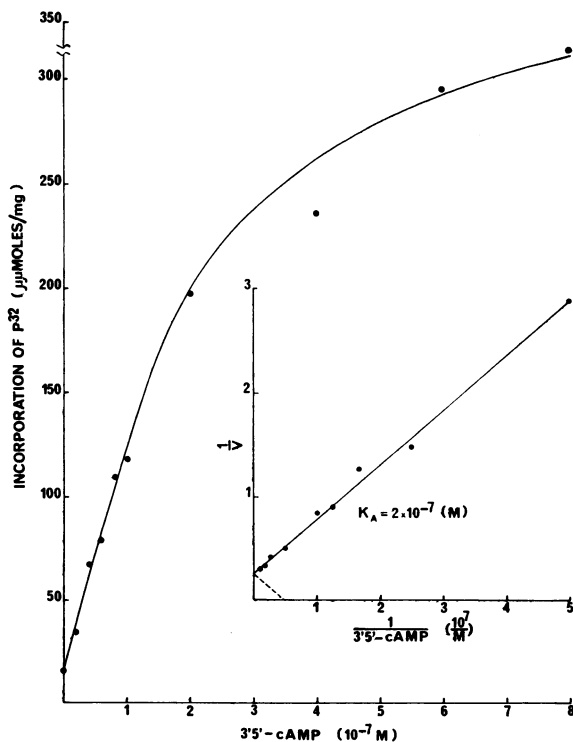


FIG. 6.—Effect of 3',5'-AMP on ^{32}P -incorporation into a PDH-phosphatase preparation from pig heart. The incubation mixture contained, in a final volume of 80 μl , pH 7.0, 30 μl of the phosphatase (102 μg), 10 μl of a 8 mM MgCl_2 solution, 20 μl of a 144 μM solution of γ - ^{32}P -ATP (1.67×10^6 cpm), 10 μl of a 16 mM solution of aminophylline, and 10 μl of a 3',5'-cyclic AMP solution at the concentrations indicated. After 15 min of incubation at 25°C, protein-bound radioactivity was determined in 10- μl samples. The enzyme preparation used in this experiment was from the pH 5.6 supernatant (see Table 1).

the effect of temperature upon the initial rate of the phosphorylation reaction. In the temperature range from 0° to 37°C, rate of incorporation increased. The temperature coefficient was about 2, that is that expected for a catalytic reaction.

Discussion. The experiments reported here support our previously expressed contention that inactivation and reactivation of heart muscle PDH are related with phosphorylation and dephosphorylation of the enzyme protein. In this respect, heart muscle PDH and the PDH complex from beef kidney³ appear to behave alike. However it should be noted that the heart muscle enzyme, even after extensive purification, was present only in part as active (dephospho) enzyme. Only after preincubation with a sufficiently high concentration of Mg^{++} and the phosphatase preparation did the highly purified PDH gain full activity. Recent as-yet-unpublished studies in our laboratory indicate that in the rat heart the ratio of active to inactive PDH decreases during starvation.¹¹ These findings are of interest because they suggest a biological role of the PDH interconversion reaction. Moreover, they could explain differences in enzyme activities reported from different laboratories.

We were able to prepare the PDH phosphatase essentially free of PDH and PDH free of phosphatase. Since the PDH isolated by gradient centrifugation was still inactivated by ATP this preparation must still contain the PDH kinase. 3',5'-cyclic AMP not only enhanced the rate of phospho-PDH reactivation in the presence of ATP, but also stimulated the incorporation of ³²P from γ -³²P-ATP into the fraction containing the phosphatase. This raises the question whether the PDH-phosphatase also is phosphorylated and dephosphorylated. The phospho-phosphatase would then represent the active form of the enzyme, whereas the reverse would apply to PDH. A phosphorylation-dephosphorylation cycle of the phosphatase would also require a phosphophosphatase dephosphorylating enzyme. The interconversion of the PDH-phosphatase and its relation to the interconversion of the PDH needs to be studied. Until we have additional results, a discussion of a possible interconversion cascade of PDH and of the role that cyclic AMP may have in that interconversion cascade, seems premature. Several 3',5'-cyclic AMP dependent kinases catalyzing the phosphorylation of various proteins such as histone, casein, protamine, phosphorylase b kinase, and UDPG-glycogen glucosyl-transferase I-kinase have been reported from various sources—brain, skeletal muscle, adipose tissue, liver and *E. coli*.¹²⁻¹⁷ To that list of protein kinases now one more 3',5'-cyclic AMP stimulated protein kinase may be added. This opens attractive possibilities for control of pyruvate metabolism in heart muscle by epinephrine, glucagon, or insulin caused by a mechanism similar to that involved in the control of glycogen synthesis and breakdown.¹⁸

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