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FUNCTION OF PHOSPHORYLATION SITES ON PYRUVATE DEHYDROGENASE

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SUMMARY. Evidence is presented that dephosphorylation of the three phosphorylation sites on bovine kidney pyruvate dehydrogenase by pyruvate dehydrogenase phosphatase is random. The relative rates of dephosphorylation were in the order site 2 > site 3 > site 1. Phosphorylation site 2, and possibly site 3, function, in addition to site 1, as inactivating sites. However, the presence of phosphoryl groups at sites 2 and 3 did not significantly affect the rate of dephosphorylation at site 1 or the rate of reactivation of the enzyme by the phosphatase. The rate-limiting step in the reactivation of phosphorylated pyruvate dehydrogenase is apparently the dephosphorylation at site 1.

Activity of the mammalian pyruvate dehydrogenase complex is regulated by a phosphorylation-dephosphorylation cycle (1,2). Phosphorylation and concomitant inactivation of the complex is catalyzed by a $MgATP^{2-}$-dependent kinase, and dephosphorylation and concomitant reactivation is catalyzed by a Mg^{2+}-dependent phosphatase. The site of this covalent regulation is the pyruvate dehydrogenase component of the complex. This enzyme possesses the subunit composition $\alpha_2\beta_2$ (3), it undergoes phosphorylation on three serine residues in its α subunit (4), and it apparently exhibits half-site reactivity (4,5). Tryptic digestion of the phosphorylated pyruvate dehydrogenase yields three phosphopeptides, a mono-(site 1) and a di-(sites 1 and 2) phosphorylated tetradecapeptide and a monophosphorylated nonapeptide (site 3). Phosphorylation proceeds markedly faster at site 1 than at sites 2 and 3 on the uncomplexed pyruvate dehydrogenase component, and phosphorylation at site 1 correlates closely with inactivation of the enzyme (4).

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Abbreviations used: ATPγS, adenosine 5'-O-(γ-thio)triphosphate; MOPS, 2-(N-morpholinopropane sulfonate).

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Site 1
T-1 Tyr-His-Gly-His-Ser(P)-Met-Ser-Asn-Pro-Gly-Val-Ser-Tyr-Arg

Site 2
T-2 Tyr-His-Gly-His-Ser(P)-Met-Ser-Asn-Pro-Gly-Val-Ser(P)-Tyr-Arg

Site 3
T-3 Tyr-Gly-Met-Gly-Thr-Ser(P)-Val-Glu-Arg

Randle and coworkers reported (6) that phosphorylation at sites 2 and 3, in addition to site 1, on pig heart pyruvate dehydrogenase inhibited its reactivation by pyruvate dehydrogenase phosphatase, and they proposed (7) that phosphorylation at sites 2 and 3 may mediate the inhibitory effects of diabetes and starvation on pyruvate dehydrogenase in heart muscle. In this communication we present evidence that phosphoryl groups at sites 1, 2 and 3 on bovine kidney pyruvate dehydrogenase are released in a random manner by pyruvate dehydrogenase phosphatase. The presence of phosphoryl groups at sites 2 and 3 did not significantly affect the rate of dephosphorylation at site 1 or the rate of reactivation of the enzyme. Use of phosphatase-resistant thiophosphoryl groups indicated that phosphorylation site 2, and possibly site 3, function, in addition to site 1, as inactivating sites.

MATERIALS AND METHODS

Highly purified preparations of the bovine kidney pyruvate dehydrogenase complex (sp act 18.6 μmol NADH/min/mg protein at 30°) and its component enzymes were obtained by procedures described (8) or by modifications thereof. [γ-^{32}P] ATP was purchased from ICN Pharmaceuticals and ATPγS from Boehringer Mannheim. The isolation and purification of pyruvate dehydrogenase phosphatase to apparent homogeneity will be described elsewhere.

Phosphorylation and dephosphorylation of pyruvate dehydrogenase complex. Preparations of the pyruvate dehydrogenase complex were phosphorylated to different degrees in the presence of the endogenous pyruvate dehydrogenase kinase and [γ-^{32}P]ATP (120,000 cpm/nmol) as described by Yeaman et al. (4). Phosphorylation was stopped at the desired time, determined by a pilot experiment, by adding glucose and hexokinase to scavenge the ATP. The mixtures were dialyzed overnight at 4°. The content of protein-bound phosphoryl groups was determined as described by Linn et al. (8). Components and conditions of dephosphorylation by the phosphatase are described in the figure legends.

Determination of phosphoryl groups at individual phosphorylation sites. At various stages of the dephosphorylation, 50- or 100-μl aliquots of the incubation mixtures were added to 100- or 200-μl portions of 0.3 M NH_4HCO_3, preheated to 50°, to inactivate the phosphatase. The samples were digested with trypsin, and the tryptic phosphopeptides were separated by paper electrophoresis at pH 1.9 as described (4,9). The distribution of [^{32}P]phosphoryl groups among phosphorylation sites 1, 2 and 3 was determined from the radioactivity found in the tryptic phosphopeptides (4).

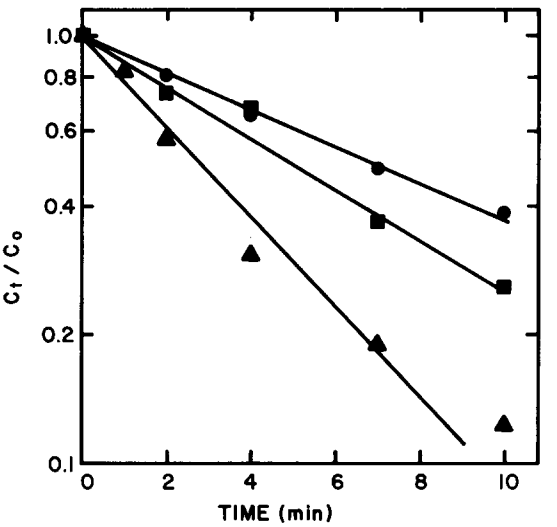

Fig. 1. Rates of dephosphorylation at individual phosphorylation sites on pyruvate dehydrogenase by pyruvate dehydrogenase phosphatase. The preparation of phosphorylated pyruvate dehydrogenase complex contained 9.7 nmol of [^{32}P]-phosphoryl groups per mg of protein. This value corresponds to 3.40 mol of phosphoryl groups per mol of the pyruvate dehydrogenase component ($\alpha_2\beta_2$) (4). The distribution of phosphoryl groups among phosphorylation sites 1, 2 and 3 was, respectively, 0.88, 0.78 and 1.7 mol per mol of $\alpha_2\beta_2$. The complex (1.0 mg/ml; equivalent to 2.86 μM $\alpha_2\beta_2$) was incubated at 30° with 3.8 μg of highly purified pyruvate dehydrogenase phosphatase, 50 mM MOPS buffer (pH 7.0), 0.1 mM $CaCl_2$ and 2 mM dithiothreitol. The dephosphorylation was started by adding 10 mM $MgCl_2$ (final concentration). Aliquots (100 μl) were withdrawn at the indicated time intervals and analyzed for content of [^{32}P]phosphoryl groups at sites 1 (●), 2 (▲) and 3 (■). C_o is the concentration at zero time of phosphoryl groups (mol per mol $\alpha_2\beta_2$) at the individual phosphorylation sites and C_t is the concentration at the time intervals indicated in the figure.

RESULTS AND DISCUSSION

The development of procedures for determining the distribution of phosphoryl groups among sites 1, 2 and 3 on phosphorylated pyruvate dehydrogenase (4) has enabled us to determine the rate of dephosphorylation at each of the three sites by pyruvate dehydrogenase phosphatase and to correlate these rates with the rates of reactivation of the phosphorylated enzyme. In most experiments a concentration of phosphorylated pyruvate dehydrogenase near the apparent K_m, about 2.9 μM (10), was used. The results presented in Fig. 1 indicate that dephosphorylation at the individual phosphorylation sites 1, 2 and 3 is first order and that site 2 and possibly site 3 are dephosphorylated at a faster rate than site 1. In several subsequent sets of experiments preparations of the

Table I

Pseudo First-Order Rate Constants for Dephosphorylation at Sites 1, 2 and 3 by Pyruvate Dehydrogenase Phosphatase

^{32}P-Labeled phosphoryl groups (mol/mol of $\alpha_2\beta_2$)				k (min^{-1})		
Total	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
1.16	0.89	0.17	0.10	0.191		
1.74	0.85	0.49	0.40	0.201	0.359	0.255
2.77	0.84	0.78	1.15	0.175	0.356	0.210

A preparation of the pyruvate dehydrogenase complex was phosphorylated to different degrees as described in Methods. Rates of dephosphorylation at sites 1, 2 and 3 were determined as described in the legend of Fig. 1. The pseudo first-order rate constants were determined by linear-regression analysis of the data.

pyruvate dehydrogenase complex were phosphorylated to different degrees, and the rates of dephosphorylation and reactivation were determined. The pseudo first-order rate constants for dephosphorylation at sites 1, 2 and 3 are given in Table I. The relative rates of dephosphorylation were in the order site 2 > site 3 > site 1. The rate constant for dephosphorylation at a specific site (e.g. site 1) was apparently not affected significantly by the presence of phosphoryl groups at the other two phosphorylation sites. We interpret these data to indicate that phosphate release from sites 1, 2 and 3 is random and, because release of phosphate from sites 2 and 3 is faster than from site 1, the rate of reactivation of phosphorylated pyruvate dehydrogenase cannot be any faster than the rate of release of phosphate from site 1. Consistent with these results is the finding of little difference, if any, in the rates of reactivation of the lightly phosphorylated (mainly at site 1) pyruvate dehydrogenase and heavily phosphorylated (at sites 1, 2 and 3) pyruvate dehydrogenase (Fig. 2A). Although the data are not shown, the initial rates of reactivation of phosphorylated pyruvate dehydrogenase complex were independent of phosphorylation at sites 2 and 3 (i.e. lightly versus heavily phosphorylated complex) when different preparations of phosphorylated complex and phosphatase

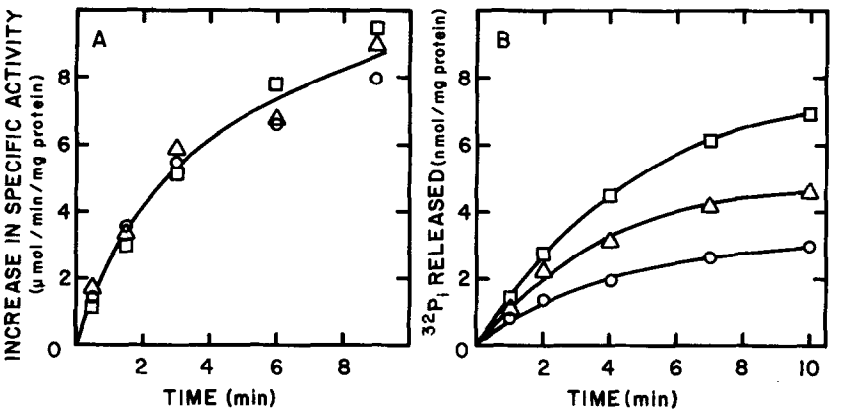

Fig. 2. Rates of (A) reactivation of and (B) release of $^{32}P_i$ from phosphorylated pyruvate dehydrogenase complex by pyruvate dehydrogenase phosphatase. The data were obtained in the same set of experiments described in Table I. The phosphorylated complexes contained 1.16 (o), 1.74 (Δ), and 2.77 (□) mol of [^{32}P]phosphoryl groups per mol of pyruvate dehydrogenase. The phosphorylated complexes possessed, respectively, about 14, 7 and 1% of the original enzyme activity. Under our conditions (4) appreciable phosphorylation at sites 2 and 3 accompanies loss of the final 25-30% of the activity of the bovine kidney and heart pyruvate dehydrogenase complexes.

were used and when the concentration of the phosphorylated pyruvate dehydrogenase component was varied between 0.71 and 28.6 μM.

The initial rate of release of total $^{32}P_i$ from the heavily phosphorylated pyruvate dehydrogenase was about 2 times the rate of release of $^{32}P_i$ from the lightly phosphorylated protein (Fig. 2B). This observation is consistent with the somewhat faster rates of dephosphorylation at sites 2 and 3 than at site 1 and the higher total concentration of phosphoryl groups in the heavily phosphorylated protein.

Sugden et al. (6) reported that the initial rate of release of $^{32}P_i$ from fully (i.e. heavily) phosphorylated pyruvate dehydrogenase complex from pig heart was approximately 2.3 times the rate of release of $^{32}P_i$ from partially (i.e. lightly) phosphorylated complex. This observation is in agreement with our data (Fig. 2B). However, Sugden et al. claim that the initial rate of reactivation of the partially phosphorylated complex was approximately 3 times the rate of reactivation of the fully phosphorylated complex. This result is in conflict with our data (Table I and Fig. 2A). Although the basis of this

Table II

Distribution of ^{32}P-Labeled Phosphoryl Groups
Among Phosphorylation Sites on Pyruvate Dehydrogenase

Enzyme	^{32}P-Labeled phosphoryl groups (mol/mol $\alpha_2\beta_2$)				Radioactivity in tryptic peptides (%)		
	Total	Site 1	Site 2	Site 3	T-1	T-2	T-3
$PDH(OP)_1$	0.99	0.82	0.10	0.07	72.6	20.7	6.7
$PDH(OP)_3$	3.28	1.18	1.11	0.99	2.2	67.5	30.2
$PDH(OP)_1(SP)_2$	0.80	0.60	0.06	0.14	1.7	81.0	17.3

Crystalline pyruvate dehydrogenase (5.9 mg) was phosphorylated and inactivated by incubation at 23° for 10 min with 0.46 mg of purified pyruvate dehydrogenase kinase, 0.125 mM [γ-^{32}P]ATP, 2 mM dithiothreitol, 0.1 mM EDTA, 10 mM NaF and 20 mM potassium phosphate buffer (pH 7.0) in a final volume of 2.0 ml. To 0.4 ml of the incubation mixture (1.18 mg of pyruvate dehydrogenase) was added 2.71 mg of dihydrolipoyl transacetylase-kinase subcomplex (8) and 1.17 mg of dihydrolipoyl dehydrogenase (to reconstitute the pyruvate dehydrogenase complex) in a final volume of 0.8 ml. Phosphorylation was allowed to continue for 1 h at 23°, and the solution was dialyzed at 4° for 16 h against 0.02 M MOPS buffer (pH 7.0), 0.5 mM dithiothreitol, and 0.1 mM EDTA to obtain the sample designated $PDH(OP)_3$. To 1.2 ml of the original incubation mixture was added, after the 10 min incubation period, 0.3 ml of 2 M glucose and 10 µg of hexokinase to scavenge the ATP. To each of two 0.5-ml aliquots were added 2.71 mg of dihydrolipoyl transacetylase-kinase subcomplex and 1.17 mg of dihydrolipoyl dehydrogenase to reconstitute the pyruvate dehydrogenase complex. To one sample of reconstituted complex was added 0.5 mM ATPγS in a total volume of 1.0 ml. This sample and a control sample devoid of ATPγS were incubated for 1 h at 23° and then dialyzed as described above to provide the samples designated $PDH(OP)_1(SP)_2$ and $PDH(OP)_1$, respectively. Aliquots were taken for tryptic digestion and paper electrophoresis to determine the distribution of radioactivity in tryptic phosphopeptides T-1, T-2 and T-3 and the distribution of [^{32}P]phosphoryl groups at sites 1, 2 and 3.

discrepancy is not known, to our knowledge there is no significant difference in the regulatory properties of the pyruvate dehydrogenase complexes from pig heart and bovine kidney and heart.

Support for our conclusion that dephosphorylation at sites 1, 2 and 3 proceeds by a random mechanism was obtained from experiments with ATPγS. The γ-thiophosphoryl group in this compound can be transferred to protein substrates by protein kinases, but the thiophosphorylated proteins are resistant to hydrolysis by phosphoprotein phosphatases (11,12). In the present investigation we took advantage of the specificity of uncomplexed pyruvate dehydrogenase kinase (4) to preferentially insert a ^{32}P-labeled phosphoryl group into site 1

of pyruvate dehydrogenase. To this preparation were added dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase to reconstitute the pyruvate dehydrogenase complex [sample $PDH(OP)_1$] and thereby facilitate insertion of ^{32}P-labeled phosphoryl groups into sites 2 and 3 [sample $PDH(OP)_3$]. Hexokinase, which is essentially inactive with ATPγS (11), and glucose were used to scavenge [γ-^{32}P] ATP, and a second portion of $PDH(OP)_1$ was incubated with ATPγS, under conditions identical to those used in preparing sample $PDH(OP)_3$, to insert thiophosphoryl groups into sites 2 and 3 [sample $PDH(OP)_1(SP)_2$]. Because the ATPγS was not radioactive, we relied on analysis of tryptic phosphopeptides (4,8) to provide evidence that a thiophosphoryl group was inserted into site 2 on the same α chain of pyruvate dehydrogenase that contained a ^{32}P-labeled phosphoryl group at site 1. Analysis of the tryptic phosphopeptides is summarized in Table II. It should be noted that before thiophosphorylation, 72.6% of the total radioactivity of sample $PDH(OP)_1$ was found in the monophosphorylated tetradecapeptide T-1. After thiophosphorylation 81% of the total radioactivity was found in the diphosphorylated tetradecapeptide T-2. This result demonstrated that a thiophosphoryl group was indeed inserted into site 2. By analogy with the control sample $PDH(OP)_3$, we assume that a thiophosphoryl group was also inserted into site 3. The data presented in Fig. 3A show that $^{32}P_i$ was released from $PDH(OP)_1(SP)_2$ at a rate about 70% of the rate of release of $^{32}P_i$ from $PDH(OP)_1$. This observation indicates that the presence of a thiophosphoryl group at site 2, and presumably at site 3, does not significantly inhibit the release of a phosphoryl group from site 1. These results are consistent with a random rather than an ordered release of phosphate from the three phosphorylation sites. Although site 1 was dephosphorylated in sample $PDH(OP)_1(SP)_2$, the dephosphorylated sample was virtually inactive (Fig. 3B). This unexpected finding indicates that phosphorylation site 2, and possibly site 3, function, in addition to site 1, as inactivating sites on pyruvate dehydrogenase. The physiological significance of this finding is not yet clear. It should be noted, however, that because the rates of dephosphorylation at sites 2 and 3 are faster than at

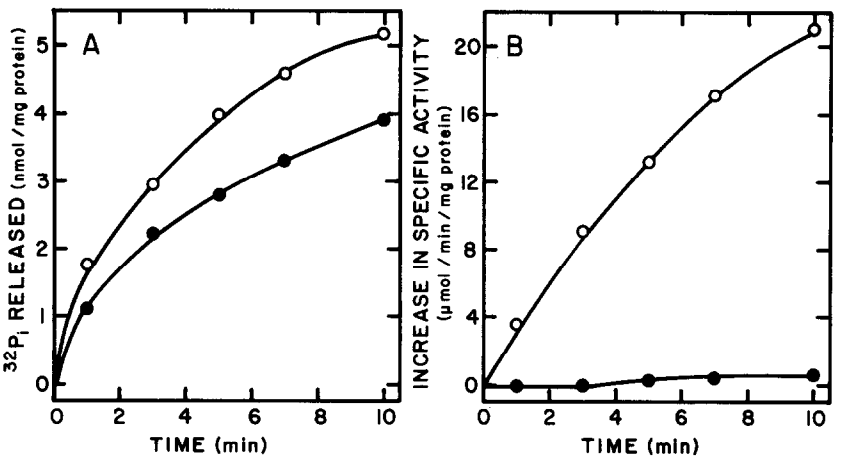

Fig. 3. Effect of thiophosphorylation at sites 2 and 3 on rates of (A) release of $^{32}P_i$ from and (B) reactivation of pyruvate dehydrogenase phosphorylated at site 1. The data were obtained in the same set of experiments described in Table II. Samples of reconstituted complex $PDH(OP)_1$ (o) and $PDH(OP)_1(SP)_2$ (•) containing 0.5 mg of pyruvate dehydrogenase were incubated at 30° with 11 μg of pyruvate dehydrogenase phosphatase as described in Fig. 1. Specific activities are based on the weight of pyruvate dehydrogenase, because an excess of dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase were used in reconstituting the pyruvate dehydrogenase complex.

site 1, the rate-limiting step in the reactivation of phosphorylated pyruvate dehydrogenase is apparently the dephosphorylation at site 1.

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REFERENCES

1. Linn, T.C., Pettit, F.H., and Reed, L.J. (1969) Proc. Nat. Acad. Sci. U.S.A. 62, 234-241.
2. Linn, T.C., Pettit, F.H., Hucho, F., and Reed, L.J. (1969) Proc. Nat. Acad. Sci. U.S.A. 62, 227-234.
3. Barrera, C.R., Namihira, G., Hamilton, L., Munk, P., Eley, M.H., Linn, T.C., and Reed, L.J. (1972) Arch. Biochem. Biophys. 148, 343-358.
4. Yeaman, S.J., Hutcheson, E.T., Roche, T.E., Pettit, F.H., Brown, J.R., Reed, L.J., Watson, D.C., and Dixon, G.H. (1978) Biochemistry 17, 2364-2370.
5. Sugden, P.H., and Randle, P.J. (1978) Biochem. J. 173, 659-668.
6. Sugden, P.H., Hutson, N.J., Kerbey, A.L., and Randle, P.J. (1978) Biochem. J. 169, 433-435.
7. Hutson, N.J., Kirbey, A.L., Randle, P.J., and Sugden, P.H. (1978) Biochem. J. 173, 669-680.
8. Linn, T.C., Pelley, J.W., Pettit, F.H., Hucho, F., Randall, D.D., and Reed, L.J. (1972) Arch. Biochem. Biophys. 148, 327-342.
9. Davis, P.F., Pettit, F.H., and Reed, L.J. (1977) Biochem. Biophys. Res. Commun. 75, 541-549.

10. Pettit, F.H., Roche, T.E., and Reed, L.J. (1972) Biochem. Biophys. Res. Commun. 49, 563-571.
11. Gratecos, D., and Fischer, E.H. (1974) Biochem. Biophys. Res. Commun. 58, 960-967.
12. Sherry, J.M.F., Gorecka, A., Aksoy, M.O., Dabrowska, R., and Hartshorne, D.J. (1978) Biochemistry 17, 4411-4418.