Peptides Derived from Pyruvate Dehydrogenase as Substrates for Pyruvate Dehydrogenase Kinase and Phosphatase

Paul F. Davis, Flora H. Pettit, and Lester J. Reed

Clayton Foundation Biochemical Institute and the Department of Chemistry
The University of Texas at Austin, Austin, Texas 78712

Received February 14,1977

SUMMARY. Evidence is presented that phosphopeptides produced by tryptic digestion of phosphorylated pyruvate dehydrogenase are effective substrates for pyruvate dehydrogenase phosphatase and that the dephosphopeptides can serve as substrates for pyruvate dehydrogenase kinase. These findings indicate that the phosphatase and the kinase do not require an intact tertiary structure in pyruvate dehydrogenase, but apparently recognize components of the local primary sequence around the phosphorylation sites.

Activity of the mammalian pyruvate dehydrogenase complex is regulated by phosphorylation and dephosphorylation catalyzed, respectively, by a $MgATP^{2-}$-requiring kinase and a Mg^{2+}-requiring phosphatase (1,2). Interconversion of PDH_a and PDH_b is modulated by ATP/ADP, acetyl-CoA/CoA, and NADH/NAD molar ratios and by the concentrations of pyruvate, Mg^{2+}, Ca^{2+}, and K^+ ions (2-9). Since the substrate for the kinase and the phosphatase is a protein, modulation of kinase and phosphatase activities could be due to binding of the various effectors to the protein substrate, to the regulatory enzyme, or to both. To distinguish between these possibilities we have sought and have found alternate substrates for the kinase and the phosphatase. Phosphopeptides produced by tryptic digestion of ^{32}P-labeled PDH_b are dephosphorylated by PDH_b phosphatase, and the dephosphopeptides are phosphorylated by PDH_a kinase and ATP. Previous studies (10,11) showed that phosphorylation occurs sequentially on three seryl residues in the α-chain of PDH. The tryptic phosphopeptides possess the amino acid sequences shown below. Phosphorylation at

Abbreviations used: PDH_a, active, nonphosphorylated form of pyruvate dehydrogenase; PDH_b, inactive, phosphorylated form.

ISSN 0006-291X

Site 1 correlates closely with inactivation of PDH (10, T.E. Roche and L.J. Reed, unpublished observations).

		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	

MATERIALS AND METHODS

Highly purified preparations of the bovine kidney pyruvate dehydrogenase complex and its component enzymes were obtained by procedures described previously (12) or by modifications thereof. Trypsin was purchased from Worthington, soybean trypsin inhibitor from Sigma, and [γ-^{32}P]ATP from ICN Pharmaceuticals. PDH_b was prepared by incubating 100 mg of PDH_a for 2 hr at 23° with 1.5 mg of kinase-transacetylase subcomplex (12), 0.05 mM [γ-^{32}P]ATP (36,000 cpm/nmol), 2 mM dithiothreitol, 1 mM $MgCl_2$, 0.1 mM EDTA and 20 mM potassium phosphate buffer (pH 7.0) in a final volume of 20 ml. The incubation mixture was dialyzed for 48 hr at 4° to remove ATP. PDH_b preparations contained 7.1-10.4 nmol of ^{32}P per mg of protein.

Digestion with trypsin. PDH_a (20 mg/ml) or PDH_b (5 mg/ml) in 0.2 M NH_4HCO_3 was incubated with trypsin (50:1 w/w) for 6 hr at 23°. A three-fold excess of soybean trypsin inhibitor was added, and the digest was lyophilized.

Purification of phosphopeptides. A tryptic digest of radioactive PDH_b was chromatographed on a column of Sephadex G-25 (fine) with 0.2 M NH_4HCO_3. The radioactive fractions were pooled, concentrated, and then subjected to paper (Whatman 3MM) electrophoresis for 45 min at pH 6.5 and 3000 V (11). The radioactive bands, located with Kodak No Screen X-ray film, were electrophoresed for 45 min at pH 1.9 and 3000 V (11). The bands were eluted with 1 M NH_4OH. In a typical purification, 0.29 µmol of peptide T-1, 0.44 µmol of T-2, and 0.09 µmol of T-3 were obtained from 1.5 µmol of PDH_b.

Dephosphorylation of phosphopeptides. The standard assay mixture contained, in a final volume of 0.35 ml, 12 nmol of ^{32}P-labeled peptide, 50 mM potassium 2-(N-morpholino)propanesulfonate buffer (pH 7.0), 2 mM dithiothreitol, 0.1 mM $CaCl_2$, and 990 units (12) of kidney PDH_b phosphatase. The reaction was started, after 2 min preincubation at 30°, by the addition of $MgCl_2$ (final concentration, 10 mM). Aliquots (50 µl) were removed at 1-, 2-, and 4-min intervals and added to 150 µl of 1 M acetic acid. The samples were chromatographed on Dowex 50X2 to separate $^{32}P_i$ and unreacted phosphopeptide as described by Nolan et al. (13). Radioactivity was determined in a Beckman LS-230 scintillation counter with ACS cocktail (Amersham/Searle).

Phosphorylation of peptides. The standard assay mixture contained, in a final volume of 0.16 ml, 20 mM phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM EDTA, uncomplexed kinase or kinase-transacetylase subcomplex, and amounts of tryptic digest of PDH_a indicated in the text. The reaction was started, after 2 min preincubation at 30°, by the addition of [γ-^{32}P]ATP (final concentration, 0.1 mM). Aliquots (50 µl) were removed at 1-, 2-, and 3-min intervals, acidified with 2 µl of 1 M HCl, and subjected to paper electrophoresis for 45 min at pH 1.9 and 3000 V. The radioactive peptides were located by radioautography, cut out, and counted as described above. Formation of phosphorylated peptide was also followed with the assay described by Carlson and Graves (14). Aliquots (25 µl) of the reaction mixtures were applied to 2.3 cm disks of Whatman P81 phosphocellulose paper. The papers were washed four times (20 min each) with 1 N acetic acid to remove ATP. The papers, which contained the phosphopeptide, were washed twice with ethanol and ether, dried, and counted.

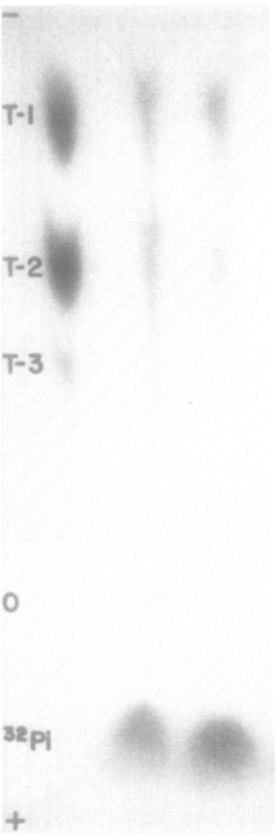

Fig. 1. Radioautograph illustrating dephosphorylation of phosphopeptides by PDH_b phosphatase. A tryptic digest of ^{32}P-labeled PDH_b (0.46 mg) was incubated at 30° with 186 units of PDH_b phosphatase and 10 mM $MgCl_2$ in a final volume of 0.14 ml as described in Methods. At 0, 5, and 10 min (left to right) 30-µl aliquots were removed and subjected to paper electrophoresis at pH 1.9 and 3000 V for 35 min.

RESULTS AND DISCUSSION

Proteolysis of PDH_b. When ^{32}P-labeled PDH_b was incubated with trypsin (50:1 w/w) for 6 hr in 0.2 M NH_4HCO_3 at 23°, essentially all of the radioactive material was soluble in 10% trichloroacetic acid. An electrophoretogram (pH 1.9) of the digest showed three radioactive spots (Fig. 1). The relative proportions of the three radioactive peptides varied with the extent of phosphorylation of PDH. Peptide T-1 is the major radioactive component in tryptic digests of lightly phosphorylated PDH (up to about 50% inactivation). As the extent of

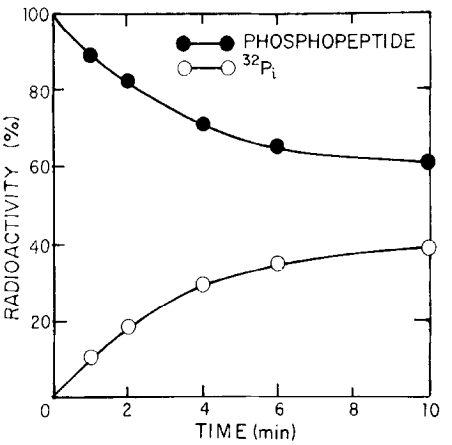

Fig. 2. Time course of dephosphorylation of peptide T-1 by PDH_b phosphatase. Components and conditions are described in Methods.

phosphorylation increased, the amount of T-1 in the tryptic digest decreased, and the amounts of T-2 and T-3 increased. In recent experiments performed by Dr. Dennis Bleile, dodecyl sulfate-polyacrylamide gel electrophoresis was used to monitor the digestion of PDH_b and PDH_a. The α-chain was attacked rapidly by trypsin, as well as by chymotrypsin and subtilisin, whereas the β-chain was degraded at a much slower rate. No activity was restored to PDH_b by limited proteolysis with these proteases at weight ratios up to 700:1.

Phosphopeptide substrates for PDH_b phosphatase. When tryptic digests of ^{32}P-labeled PDH_b were incubated with PDH_b phosphatase and Mg^{2+} and then analyzed by paper electrophoresis and radioautography, a decrease in the amounts of the radioactive peptides and the appearance of a radioactive spot corresponding to $^{32}P_i$ were observed (Fig. 1). The extent of dephosphorylation of the radioactive peptides varied with the incubation time and the amount of PDH_b phosphatase (data not shown). For subsequent experiments, the three radioactive peptides were isolated from tryptic digests of PDH_b as described in Methods. Fig. 2 shows the time course of the dephosphorylation of peptide T-1 by PDH_b phosphatase. The apparent K_m values of the phosphatase for peptides T-1, T-2,

Table I

Effects of Transacetylase, Ca^{2+} and Mg^{2+} on PDH_b Phosphatase Activity toward Tryptic Phosphopeptide T-1

Additions	Phosphatase activity (%)
None	100
Transacetylase + EGTA	103
Transacetylase + Ca^{2+}	88
No Mg^{2+}	1

The incubation mixtures contained, in a final volume of 0.35 ml, 12 nmol of ^{32}P-labeled peptide T-1, 50 mM potassium 2-(N-morpholino)propanesulfonate buffer (pH 7.0), 2 mM dithiothreitol, 990 units of kidney PDH_b phosphatase and, where indicated, 0.82 mg of transacetylase, 0.1 mM $CaCl_2$, and 2 mM ethylene glycol bis(β-aminoethyl ether) N,N′-tetraacetate (EGTA). The reaction was started by the addition of $MgCl_2$ (10 mM), and the initial rate was determined as described in Methods. In the complete incubation mixture, 0.9 nmol of $^{32}P_i$ was released/min.

and T-3 were found to be about 625, 220 and 106 μM, respectively. The V values were estimated to be about 6.4, 9.0, and 1.2 mol of $^{32}P_i$ released per min per mol of phosphatase. By comparison, the apparent K_m of the phosphatase for uncomplexed PDH_b is about 53 μM, and the V value is about 6 mol min^{-1} mol^{-1} phosphatase. These data indicate that the apparent K_m values of the phosphatase for the phosphopeptide substrates are only 2- to 12-fold higher than the apparent K_m for the protein substrate and that the V values for the peptide and protein substrates are similar.

Previous studies (5) showed that the apparent K_m of the phosphatase for PDH_b is decreased from about 58 μM to about 2.9 μM in the presence of Ca^{2+} and dihydrolipoyl transacetylase, with little change, if any, in V. Ca^{2+} does not affect phosphatase activity toward uncomplexed PDH_b, but Ca^{2+} is required to bind the phosphatase to the transacetylase. Mg^{2+} is required for PDH_b phosphatase activity with both uncomplexed PDH_b and PDH_b bound to the transacetylase. In the present investigation we observed that the transacetylase and Ca^{2+} exhibited no stimulatory effect, but rather a slight inhibitory effect, on phos-

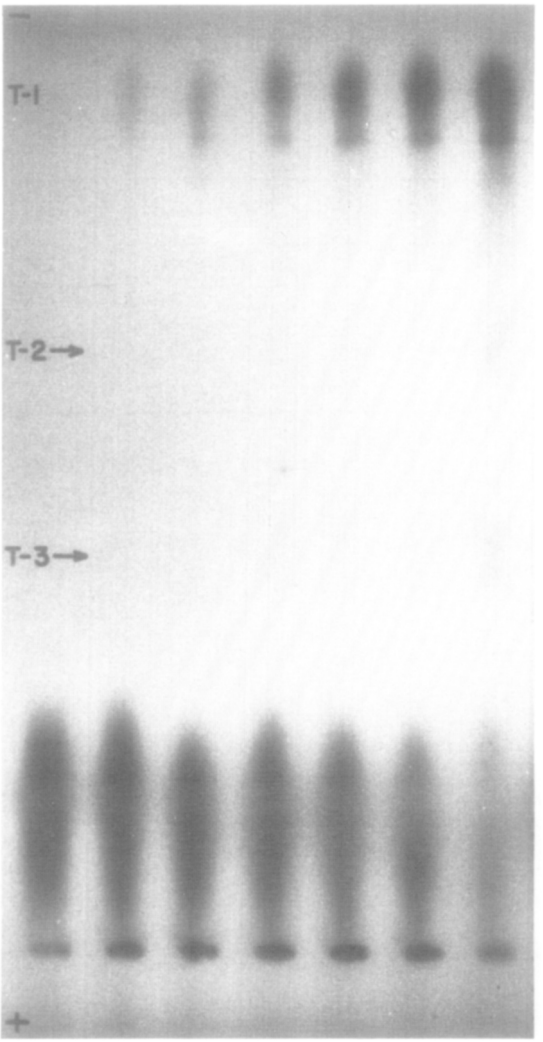

Fig. 3. Radioautograph showing time course of phosphorylation by PDH_a kinase and [γ-^{32}P]ATP of tryptic peptides derived from PDH_a. A tryptic digest of PDH_a (0.64 mg) was incubated at 30° with 92 µg of kinase-transacetylase subcomplex, 0.13 mM [γ-^{32}P]ATP, 0.13 mM $MgCl_2$, and 0.14 M NH_4HCO_3 in a final volume of 40 µl. At 0, 2, 5, 10, 20, 30, and 60 min (left to right) 5-µl aliquots were removed and subjected to paper electrophoresis at pH 1.9 and 3000 V for 45 min. Spots in ascending order represent [γ-^{32}P]ATP, peptide T-3, peptide T-2, and peptide T-1.

phatase activity toward peptide T-1 (Table I). The phosphatase showed an absolute requirement for Mg^{2+}. We interpret these results to indicate that specific binding of PDH_b and of the phosphatase (in the presence of Ca^{2+}) to the transacetylase results in favorable orientation of the phosphoseryl residues in PDH_b and the catalytic center of the phosphatase, thereby facilitating the

Mg^{2+}-dependent dephosphorylation. Since dephosphopeptide T-1 and, by inference, peptide T-1, apparently do not bind to the transacetylase (see below), the latter protein does not affect the orientation of the phosphoseryl residue in the peptide.

Peptide substrates for PDH_a kinase. In preliminary experiments purified, radioactive peptides T-1 and T-2 were dephosphorylated by incubation with PDH_b phosphatase and 10 mM $MgCl_2$ for 45 min at 30°. Control samples were removed, and the mixtures were made 10 mM with respect to NaF to inhibit the phosphatase. At 1- and 2-min intervals after addition of kinase-transacetylase subcomplex and [γ-^{32}P]ATP, aliquots were removed, acidified, and subjected, together with the control samples, to paper electrophoresis at pH 1.9, followed by radioautography. The control samples showed only trace amounts of peptides T-1 and T-2, whereas the samples that had been incubated with kinase and ATP showed a major radioactive component that migrated with the same mobility as peptide T-1. These and other observations indicate that the dephosphorylated tetradecapeptide can serve as a substrate for PDH_a kinase and that the kinase exhibits greater specificity for the seryl residue at Site 1 than at Site 2.

When a tryptic digest of PDH_a was incubated with the kinase-transacetylase subcomplex and [γ-^{32}P]ATP, and the incubation mixture was analyzed by paper electrophoresis at pH 1.9, followed by radioautography, a major and two minor radioactive peptides were detected (Fig. 3). The mobilities of the three peptides at pH 1.9 and 6.5 (data not shown) were identical with those of the three radioactive peptides detected in tryptic digests of ^{32}P-labeled PDH_b (Fig. 1). The major radioactive peptide corresponds to peptide T-1. The intensity of this spot increased with increasing incubation time (Fig. 3) and with increasing amounts of kinase-transacetylase subcomplex (data not shown). These and other data indicate that the same three seryl residues are phosphorylated in the tryptic peptides and in the protein substrate, PDH_a.

The tryptic digest of PDH_a apparently did not contain inhibitory peptides,

since the digest had little effect, if any, on kinase activity with PDH_a as substrate. The apparent K_m and V values of uncomplexed kinase for the tetradecapeptide were estimated to be about 250 μM and 1.1 mol min^{-1} mol^{-1} kinase, respectively. By comparison, the corresponding values of uncomplexed kinase for uncomplexed PDH_a are about 20 μM and 5 mol min^{-1} mol^{-1} kinase (in 20 mM phosphate buffer, pH 7.0). Previous studies (3) showed that the transacetylase markedly stimulates kinase activity with PDH_a as substrate, due apparently to specific binding and orientation of PDH_a or the kinase, or both, by the transacetylase. Thus, the apparent K_m of the kinase for PDH_a decreased from about 20 μM to about 0.6 μM in the presence of the transacetylase, and the V value increased about 2-fold. With the tetradecapeptide substrate derived from PDH_a no change in the apparent K_m was observed in the presence of the transacetylase, but the V value increased about 2.5-fold. This observation indicates that dephosphopeptide T-1 does not bind to the transacetylase. It appears that the transacetylase exerts a K_m effect on PDH_a and a V effect on the kinase. It should be noted in this connection that the conformation of PDH_a markedly affects the rate of its phosphorylation by the kinase and ATP (15).

The finding that peptides obtained by tryptic digestion of PDH_a and PDH_b can serve as substrates for PDH_a kinase and PDH_b phosphatase, respectively, is consistent with the proposal that protein kinases and phosphoprotein phosphatases recognize components of the local primary sequence around the phosphorylation site (16,17). The availability of these peptide substrates should facilitate elucidation of the site and mechanism of action of the known modifiers of PDH_a kinase and PDH_b phosphatase activities.

ACKNOWLEDGMENTS. This work was supported in part by USPHS Grant GM06590. We thank Cynthia Hook and Karen Hobson for skilled technical assistance.

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. 64, 227-234.

3. Hucho, F., Randall, D.D., Roche, T.E., Burgett, M.W., Pelley, J.W., and Reed, L.J. (1972) Arch. Biochem. Biophys. 151, 328-340.
4. Denton, R.M., Randle, P.J., and Martin, B.R. (1972) Biochem. J. 128, 161-163.
5. Pettit, F.H., Roche, T.E., and Reed, L.J. (1972) Biochem. Biophys. Res. Commun. 49, 563-571.
6. Roche, T.E., and Reed, L.J. (1974) Biochem. Biophys. Res. Commun. 59, 1341-1348.
7. Wieland, O.H., and Portenhauser, R. (1974) Eur. J. Biochem. 45, 577-588.
8. Pettit, F.H., Pelley, J.W., and Reed, L.J. (1975) Biochem. Biophys. Res. Commun. 65, 575-582.
9. Kerbey, A.L., Randle, P.J., Cooper, R.H., Whitehouse, S., Pask, H.T., and Denton, R.M. (1976) Biochem. J. 154, 327-348.
10. Hutcheson, E.T. (1971) Ph.D. Dissertation, University of Texas at Austin.
11. 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.
12. 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.
13. Nolan, C., Novoa, W.B., Krebs, E.G., and Fischer, E.H. (1964) Biochemistry 3, 542-551.
14. Carlson, G.M., and Graves, D.J. (1976) Biochemistry 15, 4476-4481.
15. Butler, J.R., Pettit, F.H., Davis, P.F., and Reed, L.J. (1977) Biochem. Biophys. Res. Commun., in press.
16. Kemp, B.E., Benjamini, E., and Krebs, E.G. (1976) Proc. Nat. Acad. Sci. U.S.A. 73, 1038-1042.
17. Daile, P., Carnegie, P.R., and Young, J.D. (1975) Nature 257, 416-418.