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Phosphorylation–Dephosphorylation of Pyruvate Dehydrogenase from Bakers' Yeast†

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ABSTRACT: The pyruvate dehydrogenase complex was purified to homogeneity from bakers' yeast (*Saccharomyces cerevisiae*). No pyruvate dehydrogenase kinase activity was detected at any stage of the purification. However, the purified pyruvate dehydrogenase complex was phosphorylated and inactivated with purified pyruvate dehydrogenase kinase from bovine kidney. The protein-bound radioactivity was localized in the pyruvate dehydrogenase α subunit. The phosphorylated, inactive pyruvate dehydrogenase complex was dephosphorylated and reactivated with purified pyruvate dehydrogenase phosphatase from bovine heart. Tryptic digestion of the ^{32}P-labeled complex yielded a single phosphopeptide, which was purified to homogeneity. The sequence of the phosphopeptide was established to be Tyr-Gly-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Thr-Thr-Tyr-Arg. This sequence is very similar to the sequence of a tryptic phosphotetradecapeptide derived from the α subunit of bovine kidney and heart pyruvate dehydrogenase: Tyr-His-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Val-Ser-Tyr-Arg.

Activity of pyruvate dehydrogenase complexes from eukaryotic sources, including mammalian, avian, and plant tissues, and *Neurospora crassa* is regulated by a phosphorylation–dephosphorylation cycle [for reviews, see Reed (1974), Denton et al. (1975), and Wieland (1983)]. Phosphorylation by pyruvate dehydrogenase kinase inactivates the complex, and dephosphorylation by pyruvate dehydrogenase phosphatase reactivates the complex. The phosphorylation sites in the mammalian pyruvate dehydrogenase complex are located on three serine residues in the α subunit of the pyruvate dehydrogenase component of the complex (Yeaman et al., 1978; Sugden et al., 1979; Mullinax et al., 1985).

Attempts thus far to demonstrate pyruvate dehydrogenase kinase activity in yeast have been unsuccessful (Kresze & Ronft, 1981a). In this paper, we present evidence that highly purified pyruvate dehydrogenase complex from bakers' yeast (*Saccharomyces cerevisiae*) undergoes phosphorylation and concomitant inactivation and dephosphorylation and concom-

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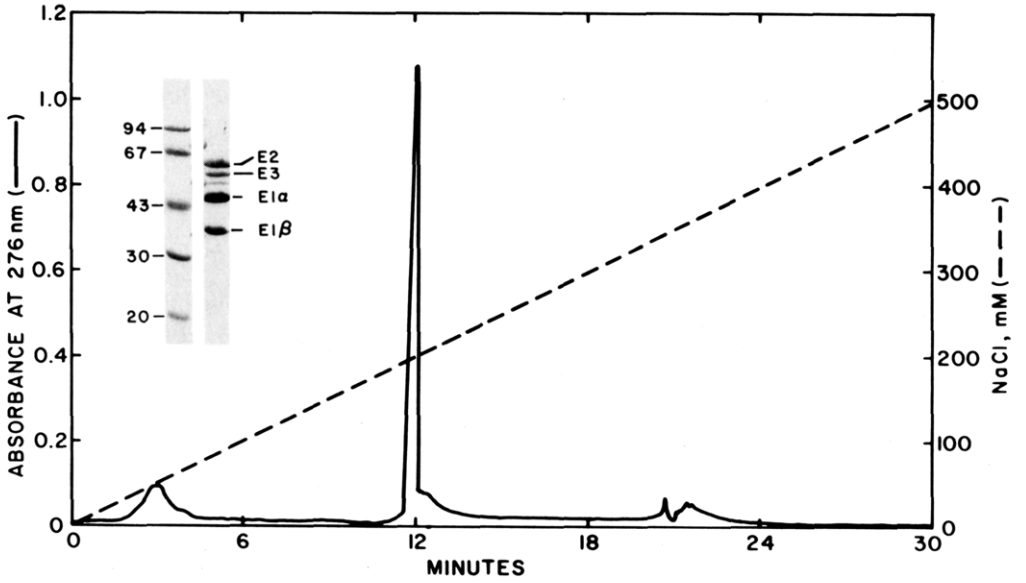

FIGURE 1: Elution profile of cation-exchange chromatography of yeast pyruvate dehydrogenase complex. About 1 mg of pyruvate dehydrogenase complex from hydroxylapatite chromatography was injected on a Custom LC (Houston, TX) Poly CAT A cation-exchange column (250 × 4.1 mm). The column was developed with a linear gradient of NaCl (0–500 mM) in 20 mM potassium phosphate buffer, pH 7.0. The flow rate was 0.75 mL/min. The pyruvate dehydrogenase complex eluted at about 185 mM NaCl. (Inset) $NaDodSO_4$–polyacrylamide gel electrophoresis pattern of highly purified pyruvate dehydrogenase complex. The gel was stained with Coomassie blue. The protein standards in $M_r \times 10^{-3}$ are, from top to bottom, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

itant reactivation, in the presence of purified pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase, respectively, from bovine kidney and heart mitochondria. We also report the amino acid sequence around the phosphorylation site.

Experimental Procedures

Materials. Pyruvate dehydrogenase kinase (Pettit et al., 1983) and pyruvate dehydrogenase phosphatase (Teague et al., 1982) were prepared from bovine kidney and heart mitochondria. The kinase used was bound to the dihydrolipoamide acetyltransferase component (E_2)[1] of the pyruvate dehydrogenase complex. The kinase comprises about 10% by weight of this subcomplex (Pettit et al., 1983). In some experiments, kinase that had been separated from E_2 was used. Fresh bakers' yeast was obtained locally. [γ-^{32}P]ATP was obtained from New England Nuclear, hydroxylapatite (high-resolution grade) was from Calbiochem, and L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin was from Worthington. All other chemicals were of the highest grade commercially available.

Assay of Overall Activity of Pyruvate Dehydrogenase Complex. The overall activity of the complex was determined by monitoring the initial rate of NADH formation at 340 nm and 30 °C with a Gilford recording spectrophotometer. The assay solution contained 2.5 mM NAD^+, 0.1 mM thiamin diphosphate, 0.13 mM coenzyme A (CoA), 1 mM $MgCl_2$, 2 mM pyruvate, 50 mM potassium phosphate (pH 8.0), and 0.32 mM dithiothreitol in a final volume of 1 mL. The pH of the solution was 7.4. The reaction was initiated by addition of enzyme. Protein was determined as described by Bradford (1976).

Measurement of protein-bound radioactivity on filter paper disks was carried out as described by Linn et al. (1972).

Amino acid analysis was performed with a Waters Pico-Tag amino acid analyzer. Peptides were hydrolyzed at 150 °C for 1 h in a reaction vial (Waters Associates) with 200 μL of 6 N HCl. Phenylthiocarbamyl amino acids were prepared according to the Waters Pico-Tag procedure with some modifications (Yang & Sepulveda, 1985).

Sequence analysis of peptides was performed according to the method of Chang et al. (1978) with a modified Edman reagent, (dimethylamino)azobenzene isothiocyanate. The (dimethylamino)azobenzene thiohydantoin amino acids were identified by either thin-layer chromatography (Chang et al., 1978; Yang, 1979) or HPLC (Yang & Wakil, 1984).

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (1970) with 15% gel and minigel apparatus. Radioactive proteins were located with Kodak XAR-5 X-ray film.

Peptide Mapping. Peptides were separated on Brinkman thin-layer cellulose plates by high-voltage electrophoresis at pH 1.9. The electrophoresis buffer consisted of acetic acid–formic acid–water (8:2:90), and electrophoresis was performed at 1000 V for 45 min.

Results

Purification of Pyruvate Dehydrogenase Complex. Unless specified otherwise, all operations were performed at 2–5 °C. *All buffers* contained 2 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, and 0.01 mM benzyloxycarbonyl-Phe-Ala diazomethyl ketone, a thiol protease inhibitor (Watanabe et al., 1979). The pyruvate dehydrogenase complex was purified from pressed bakers' yeast (0.9 kg) through steps 1–5 essentially as described by Kresze and Ronft (1981a). The pellets obtained by ultracentrifugation (step 5) were dissolved in 30 mL of 50 mM potassium phosphate buffer, pH 7.3, and the solution was applied to a column (2.5 × 10 cm) of hydroxylapatite that had been equilibrated with the same buffer. The column was washed consecutively with about 500 mL of 50, 100, and 200 mM potassium phosphate buffer, pH 7.3. The pyruvate dehydrogenase complex, detected as a yellow-green fluorescent band with a UVL-21 lamp (366 nm), was eluted with 200 mM potassium phosphate buffer con-

[1] Abbreviations: E_1, pyruvate dehydrogenase; E_2, dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; HPLC, high-pressure liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate.

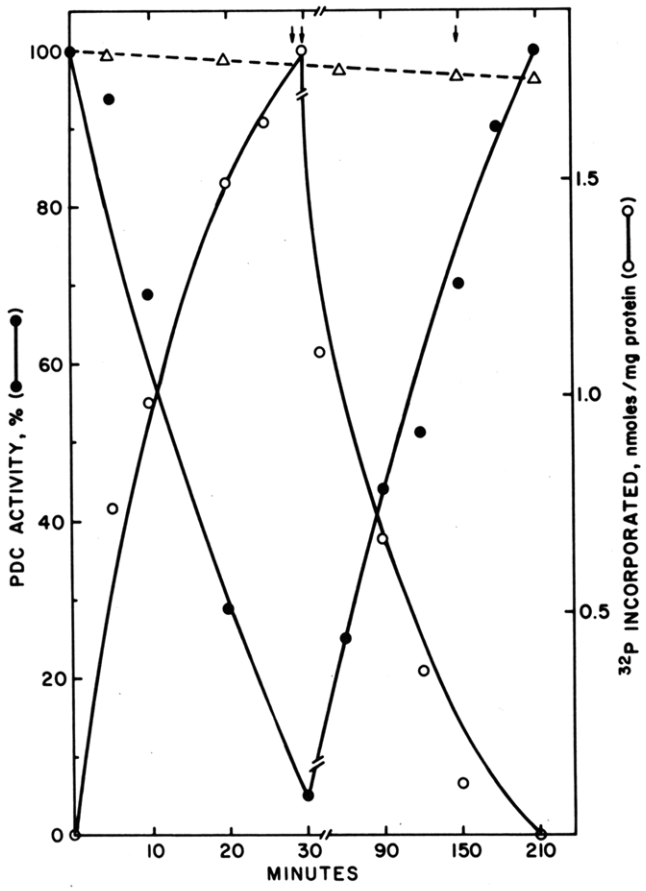

FIGURE 2: Phosphorylation/inactivation of yeast pyruvate dehydrogenase complex by bovine pyruvate dehydrogenase kinase and dephosphorylation/reactivation by bovine pyruvate dehydrogenase phosphatase. The reaction mixture contained 706 μg of highly purified pyruvate dehydrogenase complex, 5 μg of highly purified pyruvate dehydrogenase kinase from bovine kidney, 1 mM $MgCl_2$, and 0.1 mM[γ-^{32}P]ATP (230000 cmp/nmol) in 1 mL of 20 mM 3-(*N*-morpholino)propanesulfonate buffer (pH 7.2) at 30 °C. Aliquots were removed at the indicated times and assayed for pyruvate dehydrogenase complex activity and for protein-bound radioactivity. At 30 min (vertical arrow), unreacted ATP was scavenged by addition of 1 μg of hexokinase and 1 μmol of glucose. At 33 min, 5 μg of highly purified pyruvate dehydrogenase phosphatase from bovine heart and 10 mM $MgCl_2$ (final concentration) were added. Five micrograms of bovine heart phosphatase was added subsequently at 150 min.

taining 6% (w/v) ammonium sulate. The fractions containing the complex were centrifuged for 4 h at 40000 rpm in a Beckman Ti 50.2 rotor. The pellets were dissolved in 20 mM potassium phosphate buffer, pH 7.0. The yield of the pyruvate dehydrogenase complex was 90–100 mg. Specific activities were 25–30 μmol of NADH min^{-1} (mg of protein)$^{-1}$.

Homogeneous preparations of the pyruvate dehydrogenase complex were obtained by cation-exchange HPLC (Figure 1). On the basis of enzymatic activity, the recovery was about 80%. The homogeneous preparations showed four major bands on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1, inset), corresponding to pyruvate dehydrogenase ($E_1\alpha$ and $E_1\beta$), dihydrolipomide acetyltransferase (E_2), dihydrolipoamide dehydrogenase (E_3), and a minor band, $M_r \approx 50000$, of unknown function (DeMarcucci & Lindsay, 1985). The apparent M_r values of the $E_1\alpha$, $E_1\beta$, E_2, and E_3 subunits are 45000, 35000, 58000 and 56000, respectively (Kresze & Ronft, 1981b).

Phosphorylation and Dephosphorylation of Pyruvate Dehydrogenase Complex. Figure 2 illustrates phosphorylation and accompanying inactivation of highly purified yeast pyruvate dehydrogenase complex by purified pyruvate dehydrogenase kinase from bovine kidney and Mg[γ-^{32}P]ATP and dephosphorylation and accompanying reactivation of the complex upon addition of Mg^{2+} and highly purified pyruvate dehydrogenase phosphatase from bovine heart. Samples of phosphorylated, inactive pyruvate dehydrogenase complex were subjected to $NaDodSO_4$ gel electrophoresis. An autoradiograph showed that the radioactivity was localized in the $E_1\alpha$ band (Figure 3A).

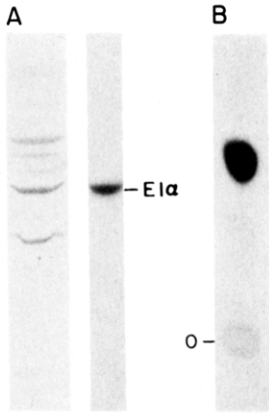

FIGURE 3: (A) Phosphorylation of yeast pyruvate dehydrogenase complex in the $E_1\alpha$ subunit. A sample (7 μg) of phosphorylated, inactive pyruvate dehydrogenase complex, from the experiment described in Figure 2, was subjected to $NaDodSO_4$ slab gel electrophoresis. The gel was stained with Coomassie blue (left) and then subjected to autoradiography (right). (B) Autoradiograph of thin-layer electrophoresis pattern of tryptic digest of ^{32}P-labeled pyruvate dehydrogenase complex. Thirty micrograms of the radioactive pyruvate dehydrogenase complex was incubated with trypsin (25:1 w/w) for 24 h in 0.2 M NH_4HCO_3 at 23 °C. The mixture was lyophilized, and the tryptic peptides were separated by electrophoresis at pH 1.9 on a cellulose thin-layer plate.

Isolation of Tryptic Phosphopeptide. In preliminary experiments, the phosphorylated pyruvate dehydrogenase complex was subjected to extensive tryptic digestion, followed by thin-layer electrophoresis at pH 1.9 and then autoradiography. The autoradiograph showed a single radioactive spot (Figure 3B). For isolation of the phosphopeptide, 21 mg of pyruvate dehydrogenase complex was incubated at 23 °C with 6 mg of E_2–kinase subcomplex from bovine kidney, 0.4 mM [γ-^{32}P]ATP (120000 cpm/nmol), 2 mM dithiothreitol, and 1 mM $MgCl_2$ in a final volume of 15 mL of 20 mM 3-(*N*-morholino)propanesulfonate buffer, pH 7.4. After 1 h of incubation of the mixture, the overall activity of the complex had decreased about 99%. To remove radioactive ATP and to change buffer, the mixture was passed, in two equal portions, through 2.5 × 15 cm columns of Sephadex G-50 preequilibrated with 0.2 M NH_4HCO_3. One milligram of trypsin was added to the phosphorylated complex, and the mixture was incubated at 23 °C for 4 h. A second 1-mg portion of trypsin was added, and incubation was continued overnight. The solution was concentrated under reduced pressure, made 2 M in urea, and applied to a Waters Associates C-18 μBondapak column (300 × 3.9 mm). The column was developed with a linear gradient (0–60%) of acetonitrile in 0.1% trifluoroacetic acid (60 min, 1.0 mL/min). A single radioactive peak was observed (data not shown). The radioactive fractions were pooled and concentrated on a vacuum centrifuge. The phosphopeptide was purified further by anion-exchange HPLC. The radioactive fractions (Figure 4) were pooled and then desalted and further purified by reverse-phase HPLC (Figure 5). The overall recovery of radioactive phosphopeptide was about 46%. The isolated material was homogeneous by chromatography and by N-terminal microsequencing. The amino acid composition

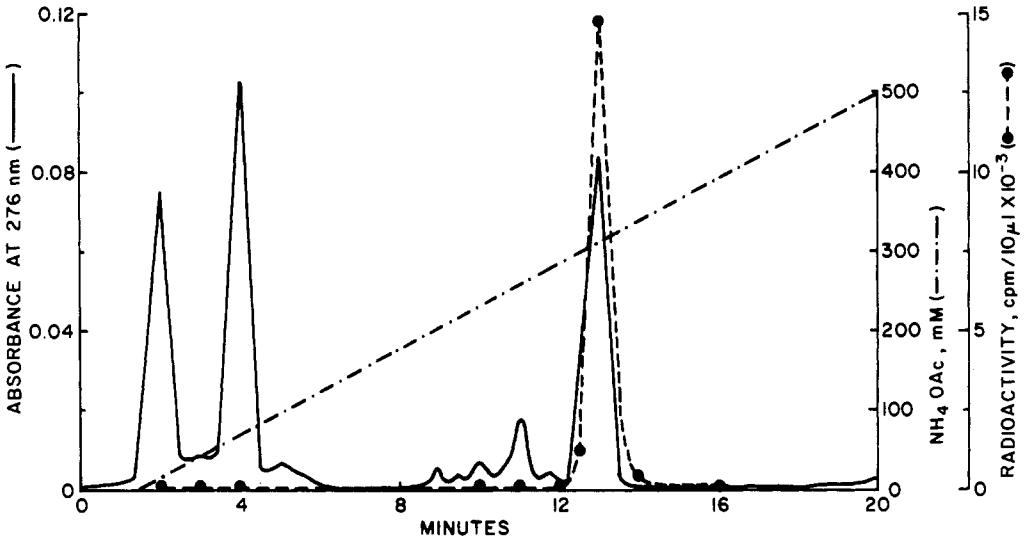

FIGURE 4: Elution profile of anion-exchange chromatography of tryptic phosphopeptide. Partially purified tryptic phosphopeptide (about 12.3 nmol) from reverse-phase HPLC was injected on a SynChropak AX-300 column (250 × 4.1 mm). The column was developed with a linear gradient of 0–1 M NH_4OAc in 20% (v/v) acetonitrile at a flow rate of 1.0 mL/min over 60 min. The radioactive peptide eluted at about 300 mM NH_4OAc. The recovery of radioactivity was about 88%.

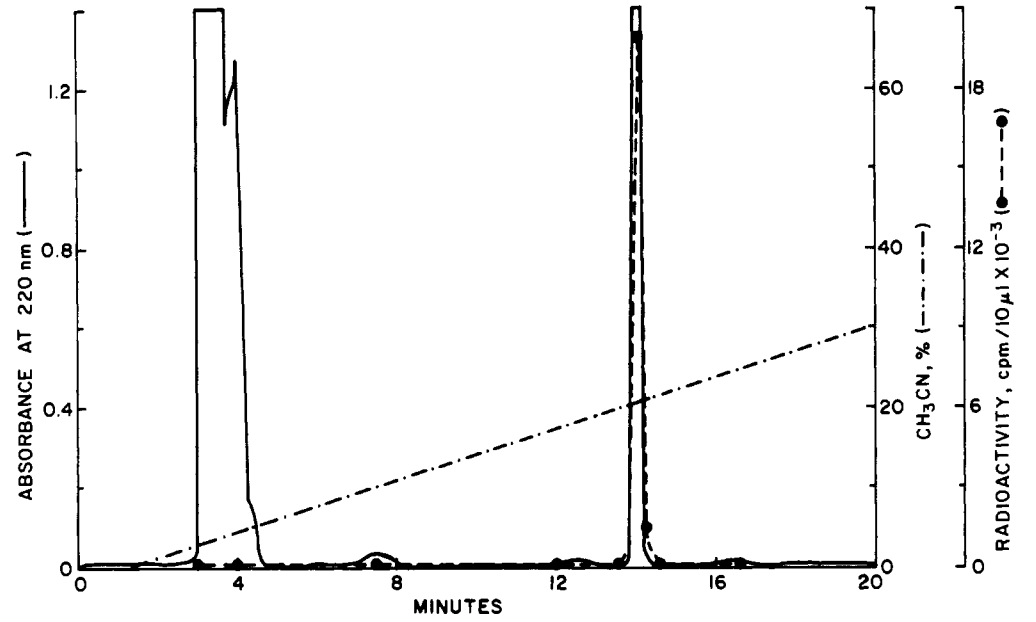

FIGURE 5: Elution profile of reverse-phase chromatography of tryptic phosphopeptide. Highly purified tryptic phosphopeptide (about 21 nmol) from anion-exchange HPLC was injected on a Waters Associates C-18 μBondapak column (300 × 3.9 mm). The column was developed with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min over 60 min. The radioactive peptide eluted at about 30% acetonitrile, and the recovery of radioactivity was essentially 100%.

of the peptide is shown in Table I.

Sequence Determination of Tryptic Phosphopeptide. The amino acid sequence of the phosphopeptide was determined on a 3.0-nmol sample by manual modified Edman degradation. All 14 amino acid residues were sequenced and identified clearly. The sequence of the phosphopeptide is presented in Table II.

For unknown reasons, release of $[^{32}P]P_i$ was not detected during the sequence degradation. Therefore, it was not possible to identify the phosphorylated residue from these data. However, limited acid hydrolysis of the phosphopeptide, followed by electrophoresis at pH 1.9 and autoradiography, showed the presence of phosphoserine (data not shown). In view of the high degree of sequence homology between the yeast tetradecapeptide and the tryptic phosphotetradecapeptide derived from the bovine kidney $E_1\alpha$ subunit, it seemed likely that the serine residue at position 5 in the yeast peptide was

Table I: Amino Acid Composition of Tryptic Phosphopeptide from Yeast Pyruvate Dehydrogenase[a]

amino acid	mol/mol of peptide	amino acid	mol/mol of peptide
Asx	1.26 (1)	Tyr	1.63 (2)
Thr	1.81 (2)	His	1.03 (1)
Ser	1.84 (2)	Arg	1.02 (1)
Pro	1.20 (1)	total	14
Gly	3.28 (3)		
Met	0.94 (1)		

[a] The amount of peptide analyzed was 0.775 nmol.

Table II: Comparison of Sequences of Phosphopeptides

Yeast	Tyr	Gly	Gly	His	Ser	Met	Ser	Asp	Pro	Gly	Thr	Thr	Tyr	Arg
Bovine[a] and	Tyr	His	Gly	His	Ser	Met	Ser	Asp	Pro	Gly	Val	Ser	Tyr	Arg
Porcine[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14

[a] From Yeaman et al. (1978) and Mullinax et al. (1985). [b] From Sugden et al. (1979).

phosphorylated. To prove this hypothesis, a sample (9 nmol) of the yeast phosphopeptide was treated with CNBr in 70% formic acid for 24 h at room temp. The solvent was removed under a stream of nitrogen, and the residue was analyzed on a SynChropak RP-P C-18 column (250 × 4.1 mm). The column was developed with a linear gradient (0–20%) of acetonitrile in 0.1% trifluoroacetic acid. A single radioactive peak eluted with a retention time of 3 min (data not shown). By comparison, the retention time for the phosphotetradecapeptide was 12 min. The amino acid composition of the former phosphopeptide, in mol/mol of peptide, was as follows: Gly (2.0), His (1.0), Tyr (0.62), Ser (0.32). A small homoserine peak was observed. These results establish that the radioactive CNBr-cleavage product is Tyr-Gly-Gly-His-Ser(P)-Hse>, and therefore, the site of phosphorylation on the tetradecapeptide is the serine residue at position 5.

Discussion

The pyruvate dehydrogenase complex is regulated by phosphorylation–dephosphorylation in all eukaryotic cells tested thus far, with the possible exception of yeast (Kresze & Ronft, 1981a). This apparent anomaly prompted us to investigate further the pyruvate dehydrogenase system in bakers' yeast. In agreement with the findings of Kresze and Ronft (1981a), no evidence was found for phosphorylation of the yeast pyruvate dehydrogenase complex in cell-free extracts or in purified preparations. However, evidence was obtained (Figure 2) that purified pyruvate dehydrogenase complex from bakers' yeast undergoes phosphorylation and inactivation in the presence of MgATP and purified pyruvate dehydrogenase kinase from bovine kidney. The phosphorylated, inactivated complex was dephosphorylated and reactivated with purified pyruvate dehydrogenase phosphatase and Mg^{2+}.

Like other eukaryotic pyruvate dehydrogenase complexes, the yeast pyruvate dehydrogenase complex contains multiple copies of four different types of polypeptide chains: $E_1\alpha$, $E_1\beta$, E_2, and E_3. The phosphorylation site on the yeast pyruvate dehydrogenase complex was localized on the $E_1\alpha$ subunit. From a tryptic digest of the phosphorylated complex, a phosphotetradecapeptide was isolated and sequenced. The sequence of this tetradecapeptide is very similar to that of a tryptic tetradecapeptide containing phosphorylation sites 1 and 2 of bovine and porcine pyruvate dehydrogenase (Table II). The yeast tetradecapeptide contains glycine instead of histidine at position 2 and threonine instead of valine and serine at positions 11 and 12. The yeast tetradecapeptide is phosphorylated on the serine residue at position 5, which corresponds to phosphorylation site 1, the major regulatory phosphorylation site on bovine and porcine pyruvate dehydrogenase. The lack of success thus far in detecting pyruvate dehydrogenase kinase activity in bakers' yeast may be due to the kinase being present in low concentrations, perhaps because it is present in an inactive form or because its biosynthesis is suppressed.

Acknowledgments

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Registry No. E_1, 9014-20-4; L-Ser, 56-45-1; pyruvate dehydrogenase kinase, 9074-01-5; pyruvate dehydrogenase phosphatase, 9073-70-5.

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