4042-4047.
Perkins, J. P., & Bertino, J. R. (1966) *Biochemistry 5*, 1005-1012.
P-L Biochemicals Inc. (1973) *UV Absorption Spectra of Pyridine Nucleotide Coenzymes and Coenzyme Analogs*, Milwaukee, Wis.
Poe, M., Greenfield, N. J., & Williams, M. N. (1974) *J. Biol. Chem. 249*, 2710-2716.
Roberts, G. C. K., Feeney, J., Burgen, A. S. V., Yuferov, V., Dann, J. G., & Bjur, R. A. (1974) *Biochemistry 13*, 5351-5357.
Schalager, L. L., & Richards, C. N. (1970) *J. Am. Chem. Soc. 92*, 5565-5570.
Shifrin, S., & Kaplan, N. O. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 177-181.
Stokes, R. H., & Robinson, R. A. (1966) *J. Phys. Chem. 70*, 2126-2130.
Udenfriend, S. (1962) *Fluorescence Assay in Biology and Medicine*, Academic Press, New York, N.Y.
Velick, S. F. (1958) *J. Biol. Chem. 233*, 1455-1467.
Wade, H. E., & Morgan, D. M. (1953) *Nature (London) 171*, 529-530.
Way, J. L., Birdsall, B., Feeney, J., Roberts, G. C. K., & Burgen, A. S. V. (1975) *Biochemistry 14*, 3470-3475.
Williams, M. N., Greenfield, N. J., & Hoogsteen, K. (1973) *J. Biol. Chem. 248*, 6380-6386.

Sites of Phosphorylation on Pyruvate Dehydrogenase from Bovine Kidney and Heart†

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ABSTRACT: The highly purified pyruvate dehydrogenase complex (EC 1.2.4.1) and uncomplexed pyruvate dehydrogenase from bovine kidney and heart mitochondria were phosphorylated and inactivated with pyruvate dehydrogenase kinase and [γ-^{32}P]ATP. Tryptic digestion of the phosphorylated pyruvate dehydrogenase yielded three phosphopeptides, a mono- (site 1) and a di- (sites 1 and 2) phosphorylated tetradecapeptide and a monophosphorylated nonapeptide (site 3). The amino acid sequences of the three phosphopeptides were established to be Tyr-His-Gly-His-Ser(P)-Met-Ser-Asn-Pro-Gly-Val-Ser-Tyr-Arg, Tyr-His-Gly-His-Ser(P)-Met-Ser-Asn-Pro-Gly-Val-Ser(P)-Tyr-Arg, and Tyr-Gly-Met-Gly-Thr-Ser(P)-Val-Glu-Arg. Phosphorylation proceeded markedly faster at site 1 than at sites 2 and 3, and phosphorylation at site 1 correlated closely with inactivation of pyruvate dehydrogenase. Complete inactivation of pyruvate dehydrogenase was associated with incorporation at site 1 of 1.0-1.6 mol of phosphoryl groups per mol of enzyme. Since pyruvate dehydrogenase is a tetramer ($\alpha_2\beta_2$) and since phosphorylation occurs only on the α subunit, the possibility of half-site reactivity is considered.

Activity of the mammalian pyruvate dehydrogenase complex is regulated by a phosphorylation-dephosphorylation cycle (Linn et al., 1969a,b). Phosphorylation and concomitant inactivation of the complex are catalyzed by a $MgATP^{2-}$-dependent kinase, and dephosphorylation and concomitant reactivation are catalyzed by a Mg^{2+}-dependent phosphatase. The site of this covalent regulation is the pyruvate dehydrogenase component of the complex. This component possesses the subunit composition $\alpha_2\beta_2$ (Barrera et al., 1972). Phosphorylation occurs on the α subunit (M_r 41 000). In this communication we present evidence that phosphorylation occurs on three serine residues (sites 1, 2, and 3) in the α subunit of bovine kidney and heart pyruvate dehydrogenase, and we report the amino acid sequences around the three phosphorylation sites. Evidence is also presented that inactivation of pyruvate dehydrogenase is associated with phosphorylation of the serine residue at site 1.

Experimental Procedure

Materials

The following materials were obtained from the sources cited: [γ-^{32}P]ATP (Amersham-Searle), L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin (Worthington); thermolysin, carboxypeptidase B, and aminopeptidase M (Sigma); carboxypeptidase Y (Pierce Chemicals); hexokinase (Boehringer-Mannheim). [γ-^{32}P]ATP was diluted at least 1:1000 with nonradioactive ATP (P-L Biochemicals). Thin-layer chromatography using ultraviolet light and radioautography for detection revealed the presence of only about 3% impurities. Highly purified preparations of the bovine kidney and heart pyruvate dehydrogenase complexes, the crystalline pyruvate dehydrogenase component, and bovine kidney PDH_a kinase were prepared as described previously (Linn et al., 1972). All other reagents and materials were of the purest grade available commercially.

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0006-2960/78/0417-2364$01.00/0

Methods

Enzyme Assays. The overall activity of the pyruvate dehydrogenase complex was determined by monitoring NADH formation at 340 nm and 30 °C (Linn et al., 1972). Activity is based on the initial rate. The activity of the pyruvate dehydrogenase component was determined after reconstituting the pyruvate dehydrogenase complex (Butler et al., 1977).

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

Phosphorylation of Pyruvate Dehydrogenase. Crystalline pyruvate dehydrogenase (154 mg; 1.0 μmol) was incubated at 23 °C with 1.5 mg of kinase–dihydrolipoyl transacetylase subcomplex from bovine kidney (Linn et al., 1972), 0.2 mM [γ-^{32}P]ATP (20 000 cpm/nmol), 2 mM dithiothreitol, 0.1 mM EDTA,[1] 1 mM $MgCl_2$, and 20 mM potassium phosphate buffer (pH 7.0) in a final volume of 20 mL. After 3 h of incubation, the mixture was dialyzed for 40 h at 4 °C against two changes of 0.2 M NH_4HCO_3 to remove ATP.

Time Course of Phosphorylation and Inactivation of the Pyruvate Dehydrogenase Complex. Bovine heart pyruvate dehydrogenase complex (6.0 mg) was incubated at 23 °C with 0.1 mM 1γ-^{32}P]ATP, 2 mM dithiothreitol, 0.1 mM EDTA, 1.0 mM $MgCl_2$, and 10 mM NaF in 2.0 mL of 20 mM phosphate buffer (pH 7.0). NaF was included to inhibit trace amounts of PDH_b phosphatase. Phosphorylation was catalyzed by the endogenous PDH_a kinase, and the reaction was started by the addition of [γ-^{32}P]ATP. Since the activity of endogenous kinase in the bovine kidney pyruvate dehydrogenase complex was about tenfold higher than that of endogenous kinase in the heart complex, the experiments with the kidney complex were carried out in the presence of ADP (ADP/ATP ratio, 4:1) to reduce the rate of phosphorylation. Aliquots (0.2 mL) were removed at the specified time intervals and added to 300 mM (final concentration) glucose and 10 μg of hexokinase in 0.1 mL to scavenge the ATP. A small aliquot (1 or 2 μL) was analyzed for NAD-reduction activity, duplicate aliquots (50 μL) were assayed for protein-bound phosphoryl groups, and the remainder of the sample was dialyzed for 40 h at 4 °C against 0.2 M NH_4HCO_3. Control experiments showed that under the conditions used hexokinase and glucose rapidly degraded the radioactive ATP and did not affect the enzymatic activity or the ^{32}P content of the complex. The dialyzed material was digested with trypsin (50:1, w/w) for 6 h at 23 °C, lyophilized, and then subjected to high-voltage paper electrophoresis at pH 1.9 for 40 min. Radioactive peptides were located with Kodak No Screen X-ray film, the spots were cut out, and radioactivity was determined in a Beckman LS-230 scintillation counter with 5-mL portions of ACS cocktail (Amersham-Searle).

The content of pyruvate dehydrogenase tetramers in the pyruvate dehydrogenase complex was determined by titrating the thiamin pyrophosphate binding sites, two per tetramer (Butler et al., 1977; Walsh et al., 1976), with thiamin thiazolone pyrophosphate (Gutowski and Lienhard, 1976; Butler et al., 1977). The bovine kidney pyruvate dehydrogenase complex contains about 20 pyruvate dehydrogenase tetramers per molecule of complex of M_r about 7×10^6 (L. Hamilton, P. Munk and L. J. Reed, unpublished data), and the bovine heart complex (M_r about 8.5×10^6) contains about 30 pyruvate dehydrogenase tetramers.

Time Course of Phosphorylation and Inactivation of Pyruvate Dehydrogenase. The crystalline pyruvate dehydrogenase component (5 mg) of the heart and kidney pyruvate dehydrogenase complexes was treated as described above, except that NaF was omitted from the reaction mixture and highly purified PDH_a kinase (200 μg) or kinase–dihydrolipoyl transacetylase subcomplex (1.6 mg) was used. Glucose and hexokinase were used, as described above, to scavenge the ATP. The NAD-reduction activity of pyruvate dehydrogenase was measured after reconstitution of the pyruvate dehydrogenase complex (Butler et al., 1977).

Peptide Mapping. Peptides were separated on Whatman No. 3MM paper by high-voltage electrophoresis at pH 1.9 or 6.5 in the first dimension and descending chromatography in the second dimension. The electrophoresis buffers consisted of acetic acid–formic acid–water (8:2:90) and acetic acid–pyridine–water (0.4:10:90), respectively, and electrophoresis was performed at 3000 V for 40 min. For chromatography, the organic phase of a 1-butanol–acetic acid–water (4:1:5) mixture was used. Where specified, the air-dried papers were dipped in cadmium–ninhydrin reagent (Dreyer and Bynum, 1967), and the color was allowed to develop at room temperature.

Amino acid analysis was performed with a Beckman Model 121 or Model 121M amino acid analyzer. The latter was used in conjunction with the automated sequence analysis. Peptides were hydrolyzed at 110 °C for 20 h in evacuated tubes with 6 N HCl containing 10 mM phenol. Values of threonine and serine were corrected for 5% and 10% destruction, respectively.

Manual sequence analysis was carried out by the subtractive Edman method (Konigsberg, 1967).

Automated sequence analysis was carried out with a Beckman 890C Sequencer (Watson et al., 1977). Fractions obtained after each cycle of the Edman reaction were first analyzed for ^{32}P using Cerenkov counting, and then divided into two portions. One portion was hydrolyzed with 47% HI AT [25 °C for 20 h to regenerate free amino acids, which were then identified by amino acid analysis. The phenylthiohydantoin derivative of norleucine (15 nmol) was added to each tube before hydrolysis, and recoveries of amino acids were standardized to the yield of norleucine. Serine was detected as alanine and asparagine as aspartic acid by this procedure. The other portion was converted to the phenylthiohydantoin amino acid by incubation with 1 N HCl at 80 °C for 10 min, extracted with ethyl acetate, and then analyzed by thin-layer chromatography (Summers et al., 1973).

Digestion of Tryptic Phosphopeptides. Purified peptide (10–400 nmol) in 0.2 mL of suitable buffer was digested with 20 to 100 μg of protease (molar ratio, 30–50:1) for 6 min to 4 h. Digestion with thermolysin, aminopeptidase M, or carboxypeptidase B was carried out at 37 °C in 0.2 M NH_4HCO_3. Digestion with carboxypeptidase Y was performed at room temperature in 0.07 M pyridine–acetate (pH 5.5). Digestion was terminated by lyophilization. The reaction products were separated by high-voltage paper electrophoresis at pH 1.9, and, where specified, were subjected to amino acid analysis.

Treatment with CNBr was performed in 70% formic acid for 24 h at room temperature. The reaction mixture was then diluted with H_2O and lyophilized.

Partial acid hydrolysis was accomplished by treatment with 2 N HCl for 10 h at 100 °C in evacuated tubes.

Results

Isolation of Tryptic Phosphopeptides. When ^{32}P-labeled PDH_b, from both bovine heart and kidney, uncomplexed or bound to the dihydrolipoyl transacetylase component in the

[1] Abbreviations used: PDH_a, active, nonphosphorylated form of pyruvate dehydrogenase; PDH_b, inactive, phosphorylated form; CNBr, cyanogen bromide; M_r, molecular weight; EDTA, (ethylenedinitrilo)-tetraacetic acid; NAD, nicotinamide adenine dinucleotide.

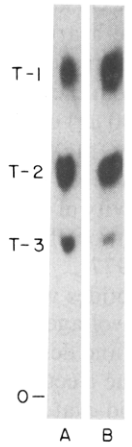


FIGURE 1: Electrophoretograms (pH 1.9) of tryptic digests of ^{32}P-labeled pyruvate dehydrogenase complex from bovine kidney (A) and heart (B). Phosphorylation of the two complexes with endogenous PDH_a kinase and [γ-^{32}P]ATP was allowed to proceed for 8 and 40 min, respectively, at 23 °C. The electrophoretic mobilities of phosphopeptides T-1, T-2, and T-3 were 0.95, 0.68, and 0.46, respectively, relative to serine (defined as 1.0).

pyruvate dehydrogenase complex, was incubated with trypsin (50:1, w/w) for 6 h in 0.2 M NH_4HCO_3 at 23 °C, essentially all of the radioactive material was soluble in 10% trichloroacetic acid. Electrophoretograms (pH 1.9) of the digests showed three radioactive spots (Figure 1). The relative proportions of the three radioactive peptides varied with the extent of phosphorylation of pyruvate dehydrogenase. Peptide T-1 was the major radioactive component in tryptic digests of lightly phosphorylated pyruvate dehydrogenase (up to about 50% inactivation). As the extent of phosphorylation increased, the amount of T-1 in the tryptic digests decreased, and the amounts of T-2 and T-3 increased (Table I).

For isolation of the phosphopeptides, 154 mg (1.0 μmol) of bovine kidney PDH_a was incubated with [γ-^{32}P]ATP and PDH_a kinase for 3 h at 23 °C as described under Methods. The phosphorylated, inactivated enzyme (1.63 μmol of phosphoryl groups/μmol of protein) was dialyzed for 40 h at 4 °C against two changes of 0.2 M NH_4HCO_3 and then digested with trypsin (50:1, w/w) for 6 h at 23 °C. The digest was lyophilized, and the residue was dissolved in 5 mL of cold 0.2 M *N*-ethylmorpholine which was adjusted to pH 8.5 with hydrochloric acid. The solution was made 10% with respect to trichloroacetic acid. After 10 min at 4 °C, the mixture was centrifuged, and the precipitate was discarded. About 90% of the radioactivity was recovered in the supernatant fluid. The solution was extracted with ether and then lyophilized. The residue was dissolved in 1.0 mL of 0.2 M NH_4HCO_3, and the solution was chromatographed on a column (100 × 2.5 cm) of Sephadex G-25 (fine). About 90% of the applied radioactivity was recovered in a broad peak (V/V_0 = 1.5). The radioactive fractions were pooled and lyophilized. The residue was subjected to paper electrophoresis at pH 1.9, and the three radioactive bands were located by radioautography. Exploratory experiments showed that each of the three radioactive bands migrated as a single radioactive component when subjected to paper electrophoresis at pH 6.5, but each band gave two radioactive components when subjected to descending paper chromatography. In each case, a sample of the faster migrating component was converted to the slower migrating component by treatment with hydrogen peroxide. These observations suggested that air oxidation of a methionine residue in peptides T-1, T-2, and T-3 had occurred. Ninhydrin staining indicated that the radioactive peptides separated by paper chromatography were essentially free of contaminating peptides. Accordingly, the three radioactive bands obtained by preparative paper electrophoresis at pH 1.9 were subjected to preparative paper chromatography, and each radioactive peptide (T-1, T-2, and T-3) and its oxidation product were eluted and combined. Analytical paper electrophoresis at pH 6.5 showed the absence of nonradioactive contaminants. In a typical isolation, 0.17 μmol of peptide T-1, 0.15 μmol of T-2, and 0.04 μmol of T-3 were recovered from a tryptic digest of 1.0 μmol of PDH_b.

The amino acid compositions of peptides T-1, T-2, and T-3 are shown in Table II. The data demonstrate that peptides T-1 and T-2 differ only in the amount of covalently bound phosphoryl groups. The former peptide contains one phosphoryl group, whereas the latter peptide contains two phosphoryl groups.

Crystalline PDH_a from bovine heart was phosphorylated and digested with trypsin, and the phosphopeptides were isolated as described above. The amino acid compositions of peptides HT-1 and HT-2 (Table II) were the same as the corresponding tryptic phosphopeptides isolated from the bovine

TABLE I: Distribution of ^{32}P in Tryptic Phosphopeptides[a]

Time (min)	Total ^{32}P (mol/mol of PDH)	Radioactivity in peptides (%)		
		T-1	T-2	T-3
0.33	0.36	74	12	14
0.67	0.61	70	14	16
1.5	0.99	64	20	16
3	1.46	53	31	16
7	2.37	33	49	18
15	3.17	17	60	23
30	3.93	8	60	32
80	4.31	5	58	37

[a] Bovine kidney pyruvate dehydrogenase complex was incubated with [γ-^{32}P]ATP. At the indicated time intervals, aliquots were removed for assay of total protein-bound phosphoryl groups and distribution of radioactivity in the three tryptic phosphopeptides derived from pyruvate dehydrogenase (PDH). Components and conditions are described under Methods.

TABLE II: Amino Acid Compositions of Tryptic Phosphopeptides from Kidney and Heart Pyruvate Dehydrogenase.[a]

Amino acid	mol/mol of peptide				
	T-1	T-2	T-3	HT-1	HT-2
Asp	1.0 (1)	1.0 (1)		1.1 (1)	1.0 (1)
Thr			1.0 (1)		
Ser	2.7 (3)	2.5 (3)	0.9 (1)	3.0 (3)	2.7 (3)
Glu			1.1 (1)		
Pro	1.0 (1)	1.0 (1)		1.0 (1)	1.3 (1)
Gly	2.1 (2)	2.1 (2)	2.1 (2)	2.2 (2)	2.3 (2)
Val	0.9 (1)	1.0 (1)	1.1 (1)	1.0 (1)	1.1 (1)
Met[b]	0.8 (1)	0.8 (1)	0.8 (1)	0.8 (1)	0.9 (1)
Tyr	1.8 (2)	1.9 (2)	0.6 (1)	1.6 (2)	1.9 (2)
His	2.3 (2)	2.2 (2)		1.8 (2)	1.9 (2)
Arg	1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)	1.1 (1)
Total	14	14	9	14	14
^{32}P	0.8 (1)	1.8 (2)	1.2 (1)	0.8 (1)	2.0 (2)

[a] Impurities below 0.2 residue are omitted. [b] Methionine determined as methionine plus methionine sulfoxide. Phosphopeptides T-1, T-2, and T-3 were derived from kidney PDH_b, and phosphopeptides HT-1 and HT-2 were from heart PDH_b.

TABLE III: Amino Acid Compositions of Subfragments Derived from Tryptic Phosphopeptides.

Amino acid	mol/mol of peptide					
	T-1A(T-2A)	T-2B	T-1B	T-2C	T-2D	T-1Th
Asp	1.6 (1)[a]				1.3 (1)	1.1 (1)
Ser	2.0 (2)	0.9 (1)	0.6 (1)	1.1 (1)	1.7 (2)	2.1 (2)
Pro		(1)[b]			1.3 (1)	(1)[b]
Gly	1.1 (1)	1.1 (1)	2.2 (1)[c]	1.3 (1)	1.4 (1)[c]	1.8 (2)
Val		0.8 (1)			0.5 (1)	
Met	0.6 (1)		1.2 (1)[d]	1.0 (1)[d]		0.7 (1)
Tyr	1.0 (1)	1.3 (1)	0.4 (1)	0.8 (1)	0.6 (1)	1.3 (1)
His	2.2 (2)		1.6 (2)	1.9 (2)		2.2 (2)
Arg		0.8 (1)			1.0 (1)	
Total	8	6	6	6	8	10
^{32}P	0.8 (1)	0.8 (1)	1.0 (1)	0.9 (1)	0.9 (1)	1.1 (1)

[a] The high value for aspartic acid is apparently due to overlap of the aspartic acid and methionine sulfoxide peaks under the conditions used in the amino acid analysis (Hutcheson, 1971). [b] Proline was estimated by comparison of the heights of the 440-nm absorption peaks of proline and serine. [c] The high values for glycine are presumably due to contamination with glycine eluted from the paper. [d] Methionine determined as hemoserine.

kidney enzyme. Although the amount of phosphopeptide HT-3 obtained was insufficient for amino acid analysis, its characteristics observed during the purification procedure were identical with those of peptide T-3 derived from bovine kidney PDH_b.

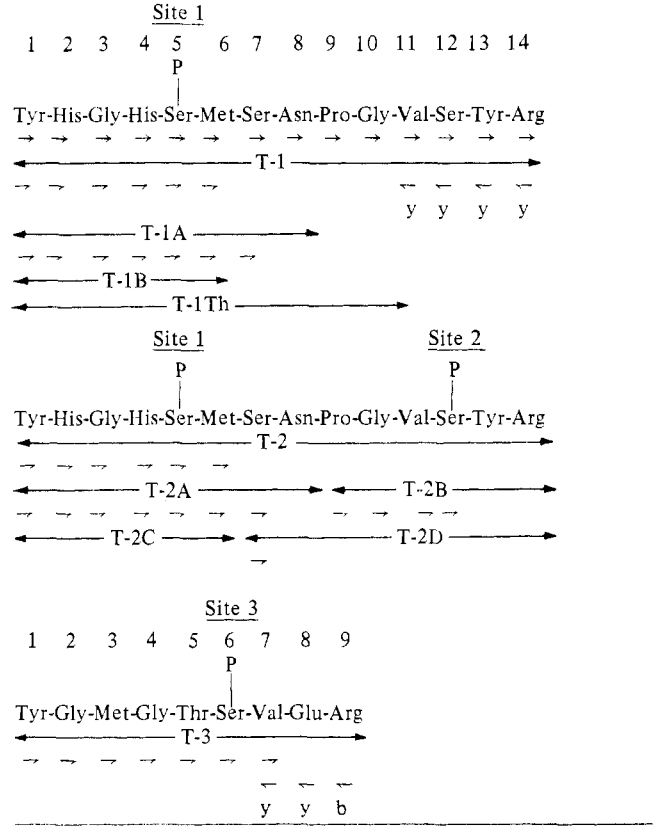
Sequence Determination of Tryptic Phosphopeptides. Manual sequence analyses using the subtractive Edman technique and quantitative amino acid analyses of subfragments derived from phosphopeptides T-1 and T-2 permitted the deduction of tentative sequences for peptides T-1, T-2, and T-3. The amino acid analyses are given in Table III, and a summary of the sequence data is shown in Table IV. The first six residues of peptides T-1 and T-2 were established by manual Edman degradation. Peptides T-1A (T2-A) and T-2B were isolated from a tryptic digest of PDH_b that had been subjected initially to ion-exchange chromatography on Aminex A-4 at 55 °C with a pH gradient of 2.5 to 5.0 (Hutcheson, 1971). The amino acid compositions of these two peptides together with Edman degradation data indicated that peptide T-1A (T-2A) corresponded to residues 1–8 of peptide T-1 (and T-2) and that peptide T-2B corresponded to residues 9–14 of peptide T-2. Peptides T-1A (T-2A) and T-2B were apparently produced by cleavage of an Asx–Pro bond in peptides T-1 and T-2 (Bradshaw et al., 1969; Piszkiewicz et al., 1970).

Automated sequence analysis of peptide T-1 confirmed and extended the earlier findings. Although no residue was identified at position 6, this residue was known to be methionine from cleavage of peptide T-1 with CNBr as described below. Residue 8 was identified as asparagine by thin-layer chromatography following conversion of the sequencer product to the phenylthiohydantoin derivative. The carboxyl-terminal sequence was confirmed by digestion of peptide T-1 with carboxypeptidase Y, which sequentially released arginine, tyrosine, serine, and valine.

The first seven residues of peptide T-3 were sequenced by the manual subtractive Edman technique. The carboxyl-terminal residue was shown to be arginine by digestion with carboxypeptidase B. Digestion with carboxypeptidase B, followed by digestion with carboxypeptidase Y, liberated arginine, glutamic acid, and then valine.

Identification of Phosphorylated Residues in the Phosphopeptides. Limited acid hydrolysis of peptides T-2 and T-3, followed by paper electrophoresis at pH 1.9 and radioautography, showed phosphoserine and orthophosphate as the only radioactive species. This observation established the site of phosphorylation on peptide T-3 as the serine residue at position 6.

TABLE IV: Summary of Sequence Data for Phosphopeptides.[a]



[a] (→) Direct Edman (automated sequencer analysis); (⇀) subtractive Edman; (←) carboxypeptidase B; (⟵) carboxypeptidase Y; Th, thermolysin.

Automated sequence analysis of peptide T-1 showed a peak of radioactivity at cycle 5 (data not shown). This observation indicated that the site of phosphorylation on peptide T-1 is the serine residue at position 5. Confirmation of this conclusion was obtained by cleavage of T-1 with CNBr. The peptide was incubated with 2 M 2-mercaptoethanol for 16 h in 0.2 M

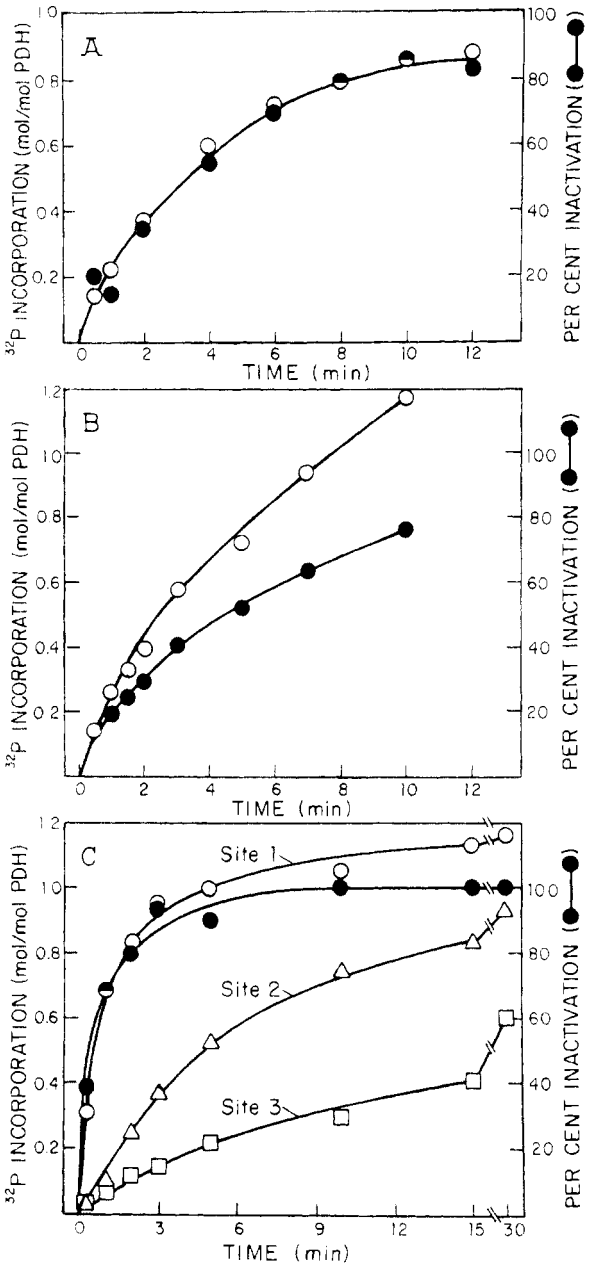


FIGURE 2: Relationship between inactivation and phosphorylation observed with different preparations of kidney pyruvate dehydrogenase. Three different preparations of crystalline pyruvate dehydrogenase (5 mg) were incubated at 23 °C with 200 μg of highly purified PDH_a kinase (A and B) or 1.6 mg of kinase-transacetylase subcomplex (C), 0.1 mM [γ-^{32}P]ATP, 1 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, and 20 mM potassium phosphate buffer (pH 7.0) in a final volume of 2.0 mL. Aliquots were withdrawn at the indicated time intervals and assayed for enzymatic activity (●), total protein-bound phosphoryl groups, and degree of phosphorylation at sites 1 (○), 2 (△) and 3 (□).

NH_4HCO_3 at 50 °C, prior to treatment with CNBr, to convert methionine sulfoxide to methionine (Polzhofer and Ney, 1971). The mercaptoethanol was removed in a vacuum. Paper electrophoresis at pH 1.9, followed by radioautography, revealed the presence of only one major radioactive peptide (T-1B). Amino acid analysis (Table III) showed that T-1B consisted of residues 1–6.

Cleavage of peptide T-2 with CNBr yielded equal amounts of two major radioactive peptides (T-2C and T-2D). The amino acid compositions of these two peptides (Table III) showed that they consisted of residues 1–6 and 7–14, respectively. Peptide T-2D was digested with aminopeptidase M, and the digest was subjected to paper electrophoresis at pH 1.9. Ninhydrin staining and radioautography showed the release of serine, but not phosphoserine or orthophosphate. Also, after one Edman degradation of peptide T-2D, essentially all of the radiolabel remained attached to the peptide. These observations demonstrate that the serine residue at position 7 in peptide T-2 is not phosphorylated. It follows, therefore, that the second site of phosphorylation of peptide T-2 is the serine residue at position 12.

Peptide T-1, but not peptides T-2 and T-3, was cleaved by thermolysin. Amino acid analysis of the single radioactive product (T-1Th, Table III), which had an electrophoretic mobility of 0.83 at pH 1.9, showed that it consisted of residues 1–10. Apparently, the presence of a phosphoryl group on the serine residue at position 12 in peptide T-2 blocked thermolysin cleavage of the Gly–Val peptide bond. Samples of peptide T-1 obtained at various stages of the phosphorylation of pyruvate dehydrogenase (see below) were cleaved quantitatively by thermolysin. This observation indicates that phosphorylation of the serine residue at position 12 occurs subsequent to, and not independently of, phosphorylation of the serine residue at position 5.

Relationship between Inactivation of Pyruvate Dehydrogenase and Phosphorylation at Site 1. A study was made of the extent of inactivation of kidney pyruvate dehydrogenase and the degree of phosphorylation at each of the three phosphorylation sites. Preparations of crystalline pyruvate dehydrogenase and the pyruvate dehydrogenase complex were phosphorylated to different degrees in the presence of PDH_a kinase and [γ-^{32}P]ATP. The degree of phosphorylation at the individual sites was calculated from the total radioactivity incorporated into pyruvate dehydrogenase and the ratio of the radioactivities found in tryptic peptides T-1, T-2, and T-3. The radioactivity at site 1 was assumed to be equal to the radioactivity found in peptide T-1 plus one-half the radioactivity of peptide T-2. The radioactivity at site 2 was assumed to be one-half the radioactivity of peptide T-2.

The results obtained with several different preparations of crystalline pyruvate dehydrogenase are shown in Figure 2. Inactivation of the pyruvate dehydrogenase tetramers ($\alpha_2\beta_2$) correlated closely with phosphorylation at site 1 on the α subunit. With uncomplexed kinase little phosphorylation, if any, at sites 2 and 3 was detected (Figure 2A,B). However, when kinase bound to dihydrolipoyl transacetylase was used, extensive phosphorylation at sites 2 and 3 occurred (Figure 2C). Presumably, binding of the kinase or the pyruvate dehydrogenase, or both, to the transacetylase facilitates phosphorylation at sites 2 and 3. With some preparations of pyruvate dehydrogenase, phosphorylation of half of the α subunits at site 1 was sufficient to block the enzymatic activity completely whereas the other half did not undergo phosphorylation (Figure 2A). However, with other preparations of pyruvate dehydrogenase complete inactivation was associated with incorporation at site 1 of more than 1.0 but less than 2.0 mol of phosphoryl groups per mol of enzyme. Preparations of crystalline pyruvate dehydrogenase from bovine heart also exhibited this latter phenomenon (data not shown).

With the bovine kidney pyruvate dehydrogenase complex, phosphorylation at site 1 proceeded at a markedly faster rate than did phosphorylation at sites 2 and 3 (Figure 3). As was observed with uncomplexed pyruvate dehydrogenase, some preparations of the kidney complex exhibited half-site reactivity with respect to phosphorylation at site 1 and inactivation (Figure 3A), whereas other preparations of the complex showed a tendency toward phosphorylation of both α subunits in the pyruvate dehydrogenase tetramers (Figure 3B).

Discussion

The data reported in this communication demonstrate that three serine residues in the α subunit of bovine kidney and heart pyruvate dehydrogenase are subject to phosphorylation by PDH_a kinase and establish the amino acid sequences around these sites (Table IV). These sequences do not bear any obvious similarities to sequences at sites on proteins which are phosphorylated by adenosine 3′:5′-monophosphate (cyclic AMP) dependent protein kinase and by other protein kinases (Kemp et al., 1975; Yeaman et al., 1977). Among a variety of other proteins tested as substrates for PDH_a kinase, only casein is phosphorylated at a detectable rate, about 0.5% of the rate observed with pyruvate dehydrogenase (Hucho et al., 1972). The only other known substrates for PDH_a kinase are the dephosphopeptides corresponding to tryptic peptides T-1 (T-2) and T-3 (Davis et al., 1977). This latter finding indicates that PDH_a kinase does not require an intact tertiary structure in pyruvate dehydrogenase but apparently can recognize components of the local primary sequence around the phosphorylation sites. However, that the conformation of pyruvate dehydrogenase plays an important role in the phosphorylation reaction is indicated by the observation that titration of the two thiamin pyrophosphate binding sites on pyruvate dehydrogenase with the transition-state analogue thiamin thiazolone pyrophosphate (Gutowski and Lienhard, 1976) strongly inhibits phosphorylation of the enzyme by PDH_a kinase and ATP (Butler et al., 1977). The conformation of the kinase is also important, as indicated by the finding that several effectors of the phosphorylation reaction appear to act by binding directly to the kinase (Pettit et al., 1975; F. H. Pettit, P. F. Davis and L. J. Reed, unpublished data).

The results presented in Figures 2 and 3 clearly show that inactivation of pyruvate dehydrogenase, uncomplexed or bound to the dihydrolipoyl transacetylase component, is associated with phosphorylation at site 1. The functional role, if any, of phosphorylation sites 2 and 3 is not known. Phosphorylation at site 2 apparently requires prior phosphorylation at site 1. However, it is not clear whether phosphorylation at site 3 requires prior phosphorylation at sites 1 and 2. Also, the location of site 3 in the primary sequence of pyruvate dehydrogenase in relation to sites 1 and 2 is not known. It should be noted that with peptide substrates derived by tryptic digestion of PDH_a, the kinase exhibits markedly greater specificity for the serine residue at site 1 than at sites 2 and 3 (Davis et al., 1977).

Although the tryptic peptides derived from heart PDH_b were not sequenced, the data presented in this communication strongly indicate that the same three sites are phosphorylated in both bovine kidney and heart pyruvate dehydrogenase. This finding is consistent with previous results, indicating that the two pyruvate dehydrogenases are very similar, if not identical (Barrera et al., 1972; Pettit et al., 1975).

Pyruvate dehydrogenase is a tetramer possessing the subunit composition $\alpha_2\beta_2$ (Barrera et al., 1972). Phosphorylation occurs on the α subunit. Some preparations of uncomplexed pyruvate dehydrogenase and of the pyruvate dehydrogenase complex clearly exhibited half-site reactivity (Lazdunski, 1974; Seydoux et al., 1974; Levitzki and Koshland, 1976) with respect to the degree of phosphorylation at site 1 and the extent of inactivation. However, with other preparations complete inactivation was associated with incorporation at site 1 of 1.2–1.6 mol of phosphoryl groups per mol of enzyme. Although the molecular basis of this difference is not known, it is possible that in some preparations of the complex not all of the 20–30 pyruvate dehydrogenase tetramers (or the subunits thereof) had a functional connection to the limited amount of dihydrolipoyl dehydrogenase (5–6 dimers). If such were the case, the degree of phosphorylation at site 1 would exceed the extent of inactivation. That the preparations of pyruvate dehydrogenase contained little, if any, protein-bound nonradioactive phosphoryl groups is indicated by the good agreement between ^{32}P content and the amino acid analyses of the phosphopeptides (Tables II and III). It should also be noted that at an early step in the isolation of the pyruvate dehydrogenase complex, the latter was exposed to PDH_b phosphatase and 10 mM Mg^{2+}, conditions which favor dephosphorylation of PDH_b.

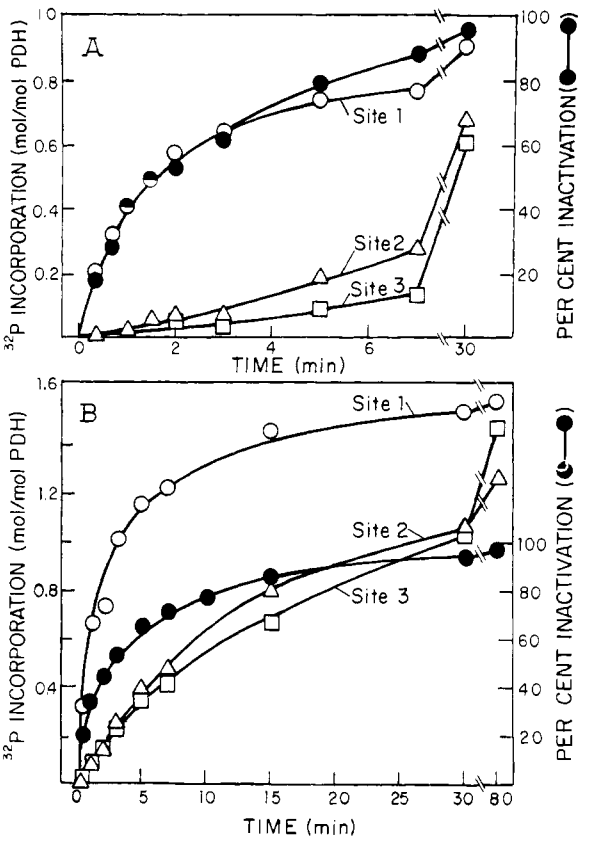


FIGURE 3: Relationship between inactivation and phosphorylation observed with different preparations of kidney pyruvate dehydrogenase complex. Two different preparations of highly purified complex (6 mg) were incubated at 23 °C with 0.1 mM [γ-^{32}P]ATP, 0.4 mM ADP, 1 mM $MgCl_2$, 0.1 mM EDTA, 10 mM NaF, 2 mM dithiothreitol, and 20 mM potassium phosphate buffer (pH 7.0) in a final volume of 2.0 mL. Aliquots were withdrawn and assayed, as described under Methods, for enzymatic activity (●), total protein-bound phosphoryl groups, and degree of phosphorylation at sites 1 (○), 2 (△), and 3 (□).

Acknowledgments

We thank Cynthia Hook and Karen Hobson for excellent technical assistance and James L. Lemburg and Dr. G. Barrie Kitto for several amino acid analyses.

Supplementary Material Available

Table on automated sequencer analysis of peptide T-1 (1 page). Ordering information is given on any current masthead page.

References

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.

Bradshaw, R. A., Babin, D. R., Nomoto, M., Srinivasin, N.

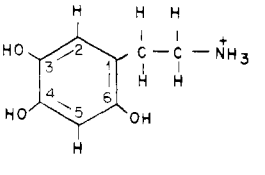
G., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1969), *Biochemistry 8,* 3859.
Butler, J. R., Pettit, F. H., Davis, P. F., and Reed, L. J. (1977), *Biochem. Biophys. Res. Commun. 74,* 1667.
Davis, P. F., Pettit, F. H., and Reed, L. J. (1977), *Biochem. Biophys. Res. Commun. 75,* 541.
Dreyer, W. J., and Bynum, E. (1967), *Methods Enzymol. 11,* 32.
Gutowski, J. A., and Lienhard, G. E. (1976), *J. Biol. Chem. 251,* 2863.
Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W., and Reed, L. J. (1972), *Arch. Biochem. Biophys. 151,* 328.
Hutcheson, E. T. (1971), Doctoral Dissertation, University of Texas at Austin.
Kemp, B. E., Bylund, D. B., Huang, T.-S., and Krebs, E. G. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3448.
Konigsberg, W. (1967), *Methods Enzymol. 11,* 461.
Lazdunski, M. (1974), *Progr. Bioorg. Chem. 3,* 81.
Levitzki, A., and Koshland, D. E., Jr. (1976), *Curr. Top. Cell. Regul. 10,* 1.
Linn, T. C., Pelley, J. W., Pettit, F. H., Hucho, F., Randall, D. D., and Reed, L. J. (1972), *Arch. Biochem. Biophys. 148,* 327.
Linn, T. C., Pettit, F. H., Hucho, F., and Reed, L. J. (1969b), *Proc. Natl. Acad. Sci. U.S.A. 64,* 227.
Linn, T. C., Pettit, F. H., and Reed, L. J. (1969a), *Proc. Natl. Acad. Sci. U.S.A. 62,* 234.
Pettit, F. H., Pelley, J. W., and Reed, L. J. (1975), *Biochem. Biophys. Res. Commun. 65,* 575.
Piszkiewicz, D., Landon, M., and Smith, E. L. (1970), *Biochem. Biophys. Res. Commun. 40,* 1173.
Polzhofer, K. P., and Ney, K. H. (1971), *Tetrahedron 27,* 1997.
Seydoux, F., Malhotra, O. P., and Bernhard, S. A. (1974), *Crit. Rev. Biochem. 2,* 227.
Summers, M. R., Smythers, G. W., and Oroszlan, S. (1973), *Anal. Biochem. 53,* 624.
Walsh, D. A., Cooper, R. H., Denton, R. M., Bridges, B. J., and Randle, P. J. (1976), *Biochem. J. 157,* 41.
Watson, D. C., Peters, E. H., and Dixon, G. H. (1977), *Eur. J. Biochem. 74,* 53.
Yeaman, S. J., Cohen, P., Watson, D. C., and Dixon, G. H. (1977), *Biochem. J. 162,* 411.

Nuclear Magnetic Resonance Studies of 6-Hydroxydopamine and Its Interactions with SH-Containing Model Compounds. Evaluation of Possible Mechanism for Neurocytotoxicity†

Joseph Granot and Avner Rotman*,‡

ABSTRACT: 6-Hydroxydopamine (I) is a well-known neurocytotoxic agent which has become an important tool in many neurochemical studies in recent years. Biochemical investigations of the mechanism of action of 6-hydroxydopamine indicated that this amine binds covalently and irreversibly to proteins. In the present work, molecular properties of 6-hydroxydopamine in aqueous solution such as self-association, ionization, intramolecular conformations, and possible cyclization were investigated using ^{1}H nuclear magnetic resonance spectroscopy. A model study for the interaction of 6-hydroxydopamine with proteins was undertaken by using SH-containing molecules: cysteine, glutathione, and bovine serum albumin. The binding of these compounds to 6-hydroxydopamine was found to cause labilization of the hydrogen attached to C_2 of the amine aromatic ring. This effect was interpreted in terms of nucleophilic attack of RS^- on C_1 of 6-hydroxydopamine. A proposed model for neurocytotoxicity is discussed.

The compound 6-hydroxydopamine (6-OHDA;[1] 2,4,5-trihydroxyphenylethylamine; I) is well-known for its selective neurotoxic action on catecholamine-containing neurons in both the peripheral and central nervous systems (Kostrzewa & Jacobowitz, 1974). This selectivity of the cytotoxic action is associated with effective uptake transport and accumulation of this compound within catecholamine neurons by the axonal amine "membrane pump", whereas the neuronal degeneration effects seem to relate to the ease of the autoxidation of 6-OHDA (Lundström et al., 1973; Sachs et al., 1975; Creveling et al., 1975). These properties have made 6-OHDA a valuable denervation tool in experimental studies in the central and the peripheral nervous system (Malmfors & Thoenen, 1971; Jonsson et al., 1975).



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Numerous data have been published concerning the mechanism of action of 6-OHDA (Rotman, 1978). It is known that, for nerve degeneration to occur, accumulation of 6-OHDA in the nerve terminals up to a critical concentration is required (Blank et al., 1972) and that autoxidation is also a prerequisite (Jonsson & Sachs, 1975). However, the specific steps resulting in the cytotoxicity are still obscure. Two possible

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[1] Abbreviations used: ^{1}H NMR, proton magnetic resonance; 6-OHDA, 6-hydroxydopamine; BSA, bovine serum albumin.

0006-2960/78/0417-2370$01.00/0