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Amino Acid Sequences around the Sites of Phosphorylation in the Pig Heart Pyruvate Dehydrogenase Complex

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1. When pig heart pyruvate dehydrogenase complex was phosphorylated to completion with [γ-^{32}P]ATP by its intrinsic kinase, three phosphorylation sites were observed. The amino acid sequences around these sites were: sequence 1, Tyr-Gly-Met-Gly-Thr-Ser(*P*)-Val-Glu-Arg; and sequence 2, Tyr-His-Gly-His-Ser(*P*)-Met-Ser-Asp-Pro-Gly-Val-Ser(*P*)-Tyr-Arg. 2. When phosphorylated to inactivation by repetitive additions of limiting quantities of [γ-^{32}P]ATP, phosphate was incorporated mainly (more than 90%) into Ser-5 of sequence 2. Phosphorylation of this site thus results in inactivation of pyruvate dehydrogenase. 3. If Ser-5 is phosphorylated with ATP and the enzyme then incubated with [γ-^{32}P]ATP, phosphorylation of the remaining sites occurred. Ser-12 of sequence 2 is phosphorylated about twice as rapidly as Ser-6 of sequence 1. 4. Incubation of pyruvate dehydrogenase with excess [γ-^{32}P]ATP with termination of phosphorylation at about 50% complete inactivation showed that Ser-5 of sequence 2 was phosphorylated most rapidly, but also that Ser-12 of sequence 2 was significantly (15% of total) phosphorylated. Ser-6 of sequence 1 contained about 1% total P. 5. These results suggest that addition of limiting amounts of ATP produces primarily phosphorylation of Ser-5 of sequence 2 (inactivating site). This also occurs during incubation with excess ATP before complete inactivation occurs, but a greater occupancy of other sites also occurs during this treatment.

Mammalian pyruvate dehydrogenase complexes contain three enzymes that catalyse the conversion of pyruvate, CoA and NAD^+ into acetyl-CoA, NADH and CO_2. The enzymes are pyruvate decarboxylase‡ (EC 1.2.4.1), lipoate acetyltransferase (EC 2.3.1.12) and lipoamide dehydrogenase (EC 1.6.4.3). [For a discussion of the enzyme nomenclature used in the present paper, see Sugden & Kerbey (1978) and Sugden & Randle (1978)]. The mammalian complex also contains an intrinsic pyruvate dehydrogenase kinase (no EC number), which catalyses an $MgATP^{2-}$-dependent phosphorylation of the α-subunit of the pyruvate decarboxylase $\alpha_2\beta_2$ tetramer, thereby inactivating the overall reaction (Linn *et al.*, 1969*a*,*b*, 1972; Barrera *et al.*, 1972; Sugden & Randle, 1978). In the pig heart complex, inactivation involves the incorporation of 1 mol of P/mol of $\alpha_2\beta_2$ tetramer (Sugden & Randle, 1978). Two further phosphorylations can then occur, without effect upon pyruvate dehydrogenase complex activity, to give a maximum incorporation of 3 mol of P/mol of $\alpha_2\beta_2$ (Sugden & Randle, 1978). These findings are comparable with those made with bovine complexes (Reed *et al.*, 1974; Davis *et al.*, 1977; Yeaman *et al.*, 1978). The non-inactivating phosphorylations are thought to be concerned with regulation of the rate of dephosphorylation and re-activation of the phospho complex by pyruvate dehydrogenase phosphate phosphatase (Hutson *et al.*, 1978; Sugden *et al.*, 1978). In the present paper we describe the amino acid sequences around the sites of phosphorylation in the α-subunit of the pig heart complex and also some studies upon the order of phosphorylation of sites in the complex under different conditions. Similar studies with the bovine complex have already been reported (Yeaman *et al.*, 1978).

‡ In the present paper, the terms pyruvate dehydrogenase and pyruvate dehydrogenase complex refer to the multienzyme holocomplex that catalyses the conversion of pyruvate, CoA and NAD^+ into acetyl-CoA, NADH and CO_2 (EC 1.2.4.1+EC 2.3.1.12+EC 1.6.4.3). The trivial name 'pyruvate decarboxylase' is used here for EC 1.2.4.1 [pyruvate–lipoate oxidoreductase (acceptor-acetylating)] in order to avoid confusion with the multienzyme holocomplex.

Experimental

Materials

Pyruvate dehydrogenase complex was purified as described by Cooper *et al.* (1974) with the modifica-

tions described in the following paper [Kerbey *et al.* (1979)]. Sources of other chemicals and reagents were as given in Cooper *et al.* (1974), Kerbey *et al.*(1976) or Sugden & Randle (1978) with the addition of the following: Worthington trypsin [treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK')] was from Cambrian Chemicals Ltd., Croydon CR9 3QL, Surrey, U.K., CNBr was from Koch–Light Laboratories Ltd., Colnbrook, Bucks. SL3 0BZ, U.K. and thermolysin was from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K. Polybrene [*NNNN'*-tetramethyl-1,6-hexanediamine polymer with 1,3-dibromopropane, hexadimethrine bromide] was from Aldrich Chemical Co., R. N. Emanuel, Wembley, Middx. HA0 1PY, U.K., X-ray film was from Kodak Ltd., Hemel Hempstead, Herts., U.K., and Whatman 3MM paper was from A. Gallenkamp and Co., London EC2P 2ER, U.K.

Methods

Pyruvate dehydrogenase complex activity. The rate of the complete reaction catalysed by the pyruvate dehydrogenase complex was measured by the direct spectrophotometric assay as described by Cooper *et al.* (1974). A unit of enzyme activity produces 1 μmol of NADH/min at 30°C.

Phosphorylation of pyruvate dehydrogenase. Four phosphorylation procedures were carried out. In each case, pyruvate dehydrogenase complex activity was measured as described above, and incorporation of ^{32}P from [γ-^{32}P]ATP (sp. radioactivity 25–40 c.p.m./pmol) into the pyruvate dehydrogenase complex was measured by the method of Corbin & Reimann (1974) as described by Sugden *et al.* (1978).

Preparation A. Pyruvate dehydrogenase complex (2000 units, 10units/ml) was phosphorylated to completion as described by Sugden *et al.* (1978) in the presence of 0.33mM-[γ-^{32}P]ATP. The incubation was for 30min at 30°C. The enzyme thus phosphorylated was 99% inactivated and contained 1.15nmol of P/unit of enzyme activity inactivated. After phosphorylation, solid urea (100g) was added to a final concentration of 6M.

Preparation B. Pyruvate dehydrogenase complex (2000 units, 10units/ml) was phosphorylated to inactivation as described by Sugden *et al.* (1978) by the repeated addition of limiting amounts of 10mM-[γ-^{32}P]ATP (nine additions of 1 μmol and then three additions of 0.5 μmol). The complete incubation took 100min and the incubation temperature was 30°C. The enzyme phosphorylated thus contained 0.38nmol of P/unit of enzyme activity inactivated and was 91% inactivated. After phosphorylation, solid urea (100g) was added to a final concentration of 6M.

Preparation C. This preparation was phosphorylated to about 95% inactivation (i.e., to about 30% complete phosphorylation) by the repetitive addition of limiting amounts of ATP. Excess ATP (and/or ADP) was removed and the enzyme was phosphorylated in the presence of excess [γ-^{32}P]ATP. Pyruvate dehydrogenase complex (1000 units, 10units/ml) was phosphorylated to inactivation by the repeated addition of limiting amounts of 10mM-ATP (nine additions of 0.5 μmol). The complete incubation took 70min and the incubation temperature was 30°C. The enzyme was 95% inactivated. At 70min, EDTA (200mM, neutralized to pH7.5 with KOH) was added to a final concentration of 10mM. The preparation was centrifuged at 150000*g* for 2h at 4°C in an MSE 75 ultracentrifuge. The pellets of pyruvate dehydrogenase phospho complex were resuspended in 20mM-potassium phosphate/2mM-dithiothreitol, pH7.0 (30ml). The enzyme was dialysed in 8/32 gauge Visking tubing for 40h against 10mM-potassium phosphate/1mM-EDTA/0.1mM-dithiothreitol, pH7.0 (2 litres) with four buffer changes. The pyruvate dehydrogenase phospho complex was recovered from the dialysis tubing (which was rinsed out with 2ml of 20mM-potassium phosphate/2mM-dithiothreitol, pH7.0). Less than 3% of the enzyme had become re-activated during dialysis. The pyruvate dehydrogenase phospho complex was then incubated at a concentration equivalent to 10 units of original activity/ml in 90ml of 20mM-potassium phosphate/2mM-dithiothreitol/2mM-$MgCl_2$/10mM-EGTA/0.3mM-[γ-^{32}P]ATP at pH7.0. Preliminary experiments showed that, under such conditions, a further incorporation (additional to the non-radioactive phosphate previously incorporated) of phosphate occurred to the extent of 0.6nmol/unit of original activity. Incorporation was complete at 30min and 50% complete at 5min. Incubation was at 30°C for 5min, after which time 100% (w/v) trichloroacetic acid was added to a final concentration of 10% (w/v). The precipitate was washed and centrifuged four times in 100ml of 10% (w/v) trichloroacetic acid. The precipitate was taken up in 20ml 0.25M-ammonium bicarbonate/8M-urea.

Preparation D. Pyruvate dehydrogenase complex (1000 units, 10units/ml) was phosphorylated as described for preparation A. The phosphorylation was terminated by the addition of 100% (w/v) trichloroacetic acid to a final concentration of 10% (w/v) when inactivation was 50% complete (30s after the initiation of the reaction by addition of [γ-^{32}P]-ATP). The precipitate was treated as described for preparation C. Incorporation of phosphate occurred to the extent of 0.50nmol/unit of enzyme activity inactivated.

Carbamoylmethylation. Pyruvate dehydrogenase phospho complex preparations were reduced and *S*-carbamoylmethylated in 6–8M urea. The pH was raised to 9 with KOH if necessary. The procedure was as described by Riley & Perham (1970), except that a similar concentration of iodoacetamide was

substituted for iodoacetic acid. Preparations were then dialysed against 125mM-ammonium bicarbonate/5mM-2-mercaptoethanol (5 litres) at 4°C to remove non-protein-bound ^{32}P. Dialysis was continued for several buffer changes until the radioactivity in the dialysis buffer had fallen to background values. The preparations were then freeze-dried or used directly for CNBr digestion.

CNBr digestion. Pyruvate dehydrogenase phospho complex or peptides derived from it were taken up in a volume (50ml) of 125mM-ammonium bicarbonate and incubated for 24h at 50°C with 2M-2-mercaptoethanol (in order to reduce any methionine sulphoxide to methionine; Polzhofer & Ney, 1971). The samples were freeze-dried, taken up in 10ml of 70% (v/v) formic acid and digested for 24h at room temperature (21°C) with CNBr/protein (1:1, w/w). Samples were diluted with 20 vol. of water and freeze-dried.

Trypsin digestion. Pyruvate dehydrogenase phospho complex, or pyruvate dehydrogenase phospho complex that had been reduced and carbamoylmethylated, and CNBr digests of the latter were dissolved in 125mM-ammonium bicarbonate/8M-urea. 125mM-Ammonium bicarbonate (3 vol.) was added. The protein content was approx. 5mg/ml. Trypsin (treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one) was added to a concentration of 0.1mg/ml. Digestion was for 1.5–6h, after which time there was no longer any 10% trichloroacetic acid-insoluble ^{32}P radioactivity.

QAE (quaternary aminoethyl)-Sephadex chromatography. Tryptic digests were diluted with an equal volume of 20mM-ammonium formate, pH8.6, and applied to a column (4cm×25cm for preparations A and B or 1.6cm×20cm for preparations C and D) of QAE-Sephadex A25 equilibrated with 20mM-ammonium formate, pH8.5. The columns were washed with 10 column vol. of 20mM-ammonium formate, pH8.6, and a linear gradient (0.02–0.6M-ammonium formate, pH8.6; total volume 4 or 2 litres) was started. Fractions (13.2 or 6.6ml) were collected and ^{32}P radioactivity determined by Čerenkov radiation. Fractions containing ^{32}P were pooled separately, diluted with distilled water (approx 1:1, v/v) and freeze-dried.

High-voltage paper electrophoresis. Peptides were dissoved in minimal volumes (0.2–1ml) of 1:1000-diluted NH_3 (sp.gr.0.88). Electrophoresis was on Whatman 3MM paper (57cm×23cm) in a Shandon Southern L24 high-voltage-electrophoresis apparatus. The following buffers were used: pH1.9 [8% (v/v) acetic acid/2% (v/v) formic acid]; pH3.5 [5% (v/v) acetic acid/0.5% (v/v) pyridine]; and pH6.5 [10% (v/v) pyridine/0.3% (v/v) acetic acid]. Electrophoresis was at 3–4kV for up to 8h, and [^{32}P]phosphopeptide migration was monitored with a Geiger counter. [^{32}P]Phosphopeptides were detected by radioautography with Kodak Blue Brand BB5 X-ray film. [^{32}P]Phosphopeptide-containing areas were sewn into Whatman 3MM paper and re-electrophoresed or eluted by descending chromatography with 0.1M-NH_3.

Gel filtration. In some cases, phosphopeptides were further purified by gel filtration on a column (1.6cm×95cm) of Sephadex G-15 equilibrated with 25mM-HCl. Fractions (1.5ml) were collected and those containing ^{32}P (as determined by Čerenkov radiation) were pooled and freeze-dried.

Amino acid analyses. Peptides were hydrolysed under N_2 at 110°C for 16h in 6M-HCl (Aristar, BDH) containing 2mg of phenol/ml. Digests were dried over solid P_2O_5/KOH and analysed on a Durrum D500 mark 2 amino acid analyser.

Automated sequence determination. This was performed as described previously (Reid, 1976; Reid & Thompson, 1978) by automated Edman degradation in a Beckman 890C automatic sequencer using a 0.1M-Quadrol [1,1′,1″,1‴-(ethane-1,2-diyldinitrilo)-tetrakis(propan-2-ol)]trifluoroacetate (pH9.5) program of Brauer *et al.* (1975). Polybrene (2mg) was added to each sample before its application to the sequencer cup to prevent loss of the sample during solvent washes in the sequencer program (Klapper *et al.*, 1978). Amino acids were identified by conversion of the thiazolinones into the amino acid phenylthiohydantoin derivatives (Reid, 1976) followed by two-dimensional t.l.c. on polyamide sheets (Summers *et al.*, 1973; Reid, 1976). Further confirmation of sequences was obtained by back-hydrolysis of the amino acid phenylthiohydantoin derivatives and subsequent amino acid analysis (Smithies *et al.*, 1971; Reid, 1976). Basic amino acids were identified by back-hydrolysis of the ethyl acetate-extracted HCl extracts after conversion of thiazolinones into phenylthiohydantoin derivatives. Other amino acids were identified by back-hydrolysis of the ethyl acetate phase.

Terminology. The first coding letter (A–D) describes the peptides isolated from pyruvate dehydrogenase phospho complex preparations A–D. The number following indicates the order of elution of peptides from the QAE-Sephadex-chromatography step. The small letters and numbers following refer to the separation of peptides in the same peak after QAE-Sephadex chromatography during high-voltage paper electrophoresis. Numbering of amino acid residues is from the *N*-terminus.

Results and Discussion

Pyruvate dehydrogenase phospho complex preparation A

The elution profile from QAE-Sephadex chromatography of a tryptic digest of pyruvate dehydrogenase phospho complex preparation A is shown in Fig. 1(*a*). Pilot studies showed that peak A4 contained

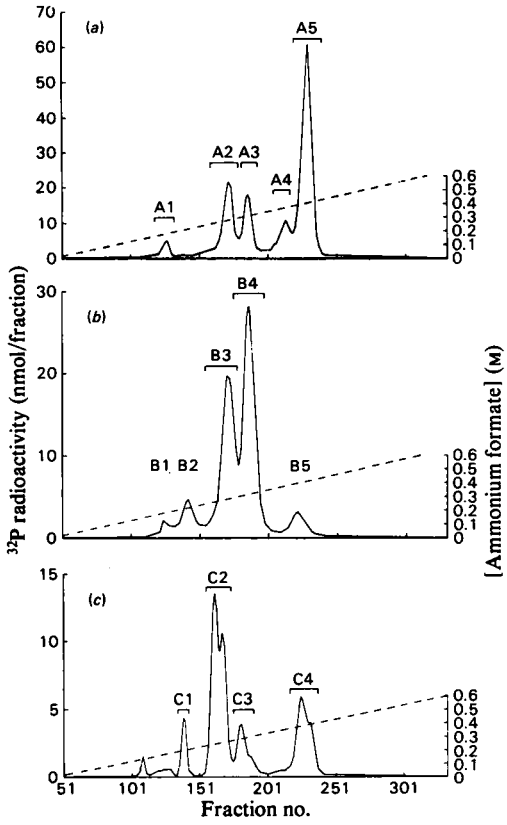

Fig. 1. *QAE-Sephadex chromatography of tryptic [^{32}P]-phosphopeptides from pyruvate dehydrogenase phosphate*

Methodology was as described in the Experimental section. Ammonium formate (----) gradients were started at fraction 51. Samples (0.5ml) were removed and counted for ^{32}P radioactivity (——). (*a*)–(*c*) Show elution of tryptic [^{32}P]phosphopeptides of pyruvate dehydrogenase phosphate preparations A–C respectively from QAE-Sephadex columns.

^{32}P-containing material that co-electrophoresed with ATP and peak A5. It was not studied further. Peak A1 was purified by electrophoresis at pH6.5 and pH3.5, peak A2 by electrophoresis at pH6.5, at pH1.9 and Sephadex G-15 gel filtration (when it was eluted at a V_e/V_0 of 1.81), peaks A3 and A5 by electrophoresis at pH6.5 and 3.5. Amino acid analyses are shown in Table 1. Peak A1 was an incomplete-digestion product. The amino acid sequence of peak A2 was determined by automated sequence analysis (Fig. 2). Serine was detected on t.l.c. of phenylthiohydantoin derivatives, but not on back-hydrolysis (where it should be identified as alanine). Some problems were encountered when attempting to determine the position of the phosphorylation site after automated sequence analysis. Dephosphorylation of the peptide occurred during sequence analysis and most (more than 95%) of the ^{32}P radioactivity remained in the cup. However, although yields were very low, 100μl samples from the 3ml butyl chloride fractions (which contains the thiazolinone derivatives) were counted for ^{32}P radioactivity in 20ml of methoxyethanol/toluene-based fluor (Severson *et al.*, 1974). Results are shown in Fig. 3(*a*). Radioactivity eluted at step 6 (corresponding to a serine residue) and at step 7 (corresponding to a valine residue). It is thus probable that the serine residue is phosphorylated in this peptide. This was confirmed by partial hydrolysis of peptide 2 (20nmol) at 110°C for 90min in 6M-HCl containing 2mg of phenol/ml. The dried residue was dissolved in water and electrophoresed at pH1.9 at 3kV for 2h. Phosphothreonine (50nmol), phosphoserine (50nmol) and [^{32}P]P_i (10000c.p.m.) were run concurrently as standards. Amino acids were detected by ninhydrin staining and ^{32}P by radioautography. At pH1.9, phosphoserine, phosphothreonine and P_i are anionic. The only [^{32}P]phospho amino acid detected was [^{32}P]phosphoserine (mobility relative to $P_i = 0.52$) and therefore Ser-6 is the residue phosphorylated by pyruvate dehydrogenase kinase. (The mobility of phosphothreonine in this system relative to P_i was 0.38.)

Peptides A3 and A5 had similar amino acid compositions (Table 1). They presumably differ in charge. Peptide A5 was sequenced by automated sequence analysis (Fig. 2). Tyrosine, glycine, methionine and valine were identified by t.l.c. of the amino acid phenylthiohydantoin derivatives. Tyrosine, histidine, glycine, methionine, aspartic acid, proline and arginine were detected by amino acid analysis of the appropriate back-hydrolysates. Serine was not detected by either t.l.c. or back-hydrolysis; its positions were assigned on the basis of there being three gaps in the amino acid sequence of peptide A5 and that amino acid analysis of peptides A5 showed the presence of three serine residues. From the appearance of ^{32}P in the butyl chloride phase after sequence analysis, the two sites of phosphorylation in peptide A5 are indicated as being Ser-5 and Ser-12 (Fig. 3). Appearance of ^{32}P at step 11 (Fig. 3*b*) is caused by a 'preview' of the following residue that was observed throughout this particular sequence analysis. Peptide A3 was also subjected to automated sequence analysis in order to determine whether the different behaviour of peptides A3 and A5 on QAE-Sephadex chromatography was caused by deamidation of Asp-8. However, the sequence of peptide A3 was identical with that of peptide A5 and hence the difference in properties is presumably caused by, e.g., difference in the methionine oxidation state.

In order to determine the positions of phosphorylation unambiguously, peptide A5 (125nmol) was reduced with 2-mercaptoethanol/ammonium bi-

Table 1. *Amino acid compositions of [^{32}P]phosphopeptides derived from pyruvate deydrogenase [^{32}P]phosphate*
Tryptic [^{32}P]phosphopeptides of pyruvate dehydrogenase preparations A–C were obtained as described in the Experimental section. The amino acids are given in nmol with the assigned no. of residues in parentheses. Notes:*, methionine was determined as the sum of methionine+methionine sulphoxide; †, methionine was determined as the sum of homoserine+homoserine lactone; ‡, the sum of nmol of peptides B3a, B4a1 and B4b1; §, the sum of nmol peptides C1 and C2a; ‖, yield calculated on the basis of original [γ-^{32}P]ATP used which in this preparation is not equivalent to nmol amino acid in C3a.

	A1	A2	A3	A5	A5a	A5a1	A5a2
Asp	12.06 (3)	—	2.96 (1)	4.90 (1)	3.02 (1)	—	—
Thr	11.30 (3)	4.72 (1)	—	—	—	—	—
Ser	10.47 (2–3)	4.07 (1)	6.59 (3)	10.61 (3)	4.64 (2)	4.36 (1)	2.57 (1)
Glu	12.20 (3)	4.96 (1)	—	1.68	—	—	—
Pro	6.58 (1–2)	—	3.01 (1)	4.29 (1)	2.41 (1)	—	—
Gly	16.29 (4)	10.09 (2)	5.97 (2)	8.46 (2)	5.85 (1)	—	—
Ala	7.51 (2)	—	—	1.04	—	—	—
Val	9.83 (2–3)	5.09 (1)	2.52 (1)	4.19 (1)	2.71 (1)	4.12 (1)	1.91 (1)
Met	4.63* (1)	4.39* (1)	0.89* (1)	3.15* (1)	—	—	—
Ile	3.42 (1)	—	—	—	—	—	—
Leu	8.22 (2)	—	—	—	—	—	—
Tyr	7.64 (2)	5.35 (1)	5.12 (2)	7.85 (2)	2.21 (1)	—	2.14 (1)
Phe	5.57 (1–2)	—	—	—	—	—	—
His	7.86 (2)	—	4.84 (2)	7.35 (2)	—	—	—
Lys	4.02 (1)	—	—	—	—	—	—
Arg	9.91 (2–3)	4.76 (1)	2.55 (1)	4.27 (1)	2.45 (1)	—	—
P	4.20 (1)	4.13 (1)	4.26 (2)	7.33 (2)	2.71 (1)	5.86 (1)	2.56 (1)
Yield (nmol of ^{32}P)	26	136	46	299	—	—	—
Total residues	33–37	9	14	14	8	2	3
Electrophoretic mobility relative to P_i at: pH6.5	0	+0.27	+0.14	+0.26	—	—	—
pH3.5	−0.30	−0.10	−0.34	−0.24	—	—	—

	B3b	B4a1	C1	C2b	C3a
Asp	3.76 (1)	—	—	7.71 (1)	—
Thr	—	—	7.55 (1)	—	—
Ser	9.01 (3)	4.89 (1)	7.34 (1)	9.28 (2)	26.42 (1)
Glu	—	—	9.61 (1)	—	—
Pro	3.09 (1)	—	—	6.62 (1)	—
Gly	7.18 (2)	6.11 (1)	8.12 (1)	7.53 (1)	34.01 (1)
Ala	—	—	—	—	—
Val	3.64 (1)	—	8.29 (1)	7.47 (1)	—
Met	—	4.75 (1)†	—	—	23.30 (1)†
Ile	—	—	—	—	—
Leu	—	—	—	—	—
Tyr	6.45 (2)	4.58 (1)	—	7.31 (1)	32.19 (1)
Phe	—	—	—	—	—
His	6.05 (2)	9.85 (2)	—	—	51.53 (2)
Lys	—	—	—	—	—
Arg	3.25 (1)	—	7.93 (1)	6.96 (1)	—
P	2.43 (1)	4.84 (1)	5.30 (1)	5.17 (1)	4.83 ‖
Yield (nmol of ^{32}P)	72	112‡	23§	43	7.2 ‖
Total residues	13/14	6	6	8	6

carbonate (2ml) under N_2, dried over solid P_2O_5/KOH, redissolved in 70% (v/v) formic acid and incubated with CNBr (20mg). After removal of formic acid/CNBr, the residue was dissolved in 125mM-ammonium bicarbonate (1ml) and treated with 0.6mg of thermolysin (thermolysin/original protein, approx. 1:50, w/w) for 24h at 37°C. The digest was chromatographed on a column (1.6cm × 35cm) of Sephadex G-25 medium grade equilibrated with 25mM-HCl. Fractions containing ^{32}P (V_e/V_0 = 1.83) were pooled, freeze-dried, redissolved in 20mM-NH_3 and a sample electrophoresed at pH3.5. The ^{32}P-containing material was eluted with 6M-HCl containing 2mg of phenol/ml. Two peptides of

Sequence 1 (peptide A2)

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 1   2   3   4   5   6       7   8   9
Tyr-Gly-Met-Gly-Thr-Ser(P)-Val-Glu-Arg
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Sequence 2 (peptides A3 and A5)

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 1   2   3   4   5       6   7   8   9  10  11  12      13  14
Tyr-His-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Val-Ser(P)-Tyr-Arg
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Fig. 2. *Amino acid sequences around the sites of phosphorylation in pyruvate dehydrogenase phosphate*
Sequences and sites of phosphorylation were determined as described in the Experimental, and Results and Discussion, sections.

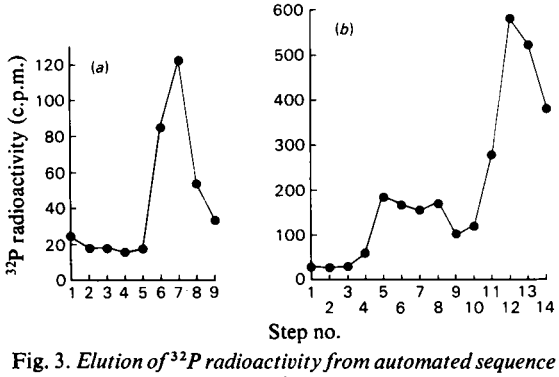

Fig. 3. *Elution of ^{32}P radioactivity from automated sequence analysis*
In (*a*), 0.1ml of the butyl chloride wash (3ml total vol.) from each automatic-sequence-analysis step for peptide A2 (sequence 1) was counted for radioactivity in 20ml of methoxyethanol/toluene-based scintillant (Severson *et al.*, 1974). In (*b*), the butyl chloride wash from automatic-sequence-analysis steps for peptide A5 (sequence 2) was evaporated to dryness and taken up in 1M-HCl (200μl). A sample (10μl) was counted for radioactivity as described above.

similar amino acid compositions were recovered. One of these (peptide A5a) is described in Table 1. Peptide A5a apparently corresponds to residues 7–14 of peptide A5, but contains contaminating material eluting with glycine. The Gly–Val bond was not cleaved by thermolysin (24h at 37°C; thermolysin/original protein 1:50, w/w), presumably because Ser-12 is phosphorylated (see Yeaman *et al.*, 1978). Cleavage at Met-6 was successful. It is presumed that residues 1–6 of peptide A5 were lost during this treatment, possibly during gel filtration, because of aromatic-adsorption effects. However, this evidence supports the conclusions that it is residues 5 and 12 that are phosphorylated.

A portion of peptide A5a (80nmol) was subjected to partial acid hydrolysis in 6M-HCl containing 2mg of phenol/ml at 100°C for 45min under N_2. After drying and redissolving the hydrolysate, electrophoresis at pH3.5 showed several bands of ^{32}P-containing material. The major bands co-electrophoresed with unhydrolysed peptide, phosphoserine and P_i. Two other major peptides (A5a1 and A5a2) were isolated from the partial hydrolysate, namely Val-Ser(*P*) and Val-Ser(*P*)-Tyr (Table 1). The evidence, together with the ratio of phosphate to amino acids in peptide A5a, confirms that the sites of phosphorylation in peptide A5 are serine residues 5 and 12.

The amino acid sequences of the sites of phosphorylation in the pyruvate dehydrogenase complex from pig heart are similar to those seen in the bovine heart or kidney enzyme (Yeaman *et al.*, 1978) except that for peptide A5, Asp-8 in the pig heart enzyme is Asn-8 in the bovine enzyme. It seems unlikely that deamidation of the peptide could have occurred under the mild conditions used. The finding that peptide A3 (identical with A5) also contains Asp-8 also supports this conclusion. That the difference in amino acid sequence at residue 8 between the bovine and pig enzymes is a real species difference is supported by consideration of the codons for aspartic acid (GAU, GAC) and asparagine (AAU, AAC). Only a single purine transition is required for this difference to occur. The sequence around the phosphorylation sites is different from that around the phosphorylation sites observed in substrates of cyclic AMP-dependent protein kinase (for a review, see Nimmo & Cohen, 1977). The only specificity observed with pyruvate dehydrogenase kinase is that there is a glycine residue one residue removed on the *N*-terminal side of the serine residue phosphorylated. It has previously been shown (Davis *et al.*, 1977) that the bovine kidney dephosphopeptides described above are substrates for pyruvate dehydrogenase kinase. This implies that the primary sequence of the peptides plays a role in their recognition by the kinase. In this regard, the sequence Gly-X-Ser may be important. It should also be recognized that for all protein kinases, primary sequence is not the only

factor involved in determining whether a particular hydroxy group is phosphorylated. The hydroxy group must also be exposed to the kinase enzyme and not hidden within the protein substrate itself. Two factors therefore regulate protein phosphorylation, namely primary sequence and hydroxy-group exposure.

Pyruvate dehydrogenase phosphate preparation B

The elution profile from QAE-Sephadex chromatography of a tryptic digest of CNBr-treated pyruvate dehydrogenase phosphate preparation B is shown in Fig. 1(*b*). Since fractions B3 and B4 represented about 87% of ^{32}P radioactivity, only these were purified. Peak-B3 material was purified by electrophoresis at pH 6.5 and pH 1.9 when three bands were observed (B3a–B3c). Material from bands B3a and B3b was further purified by Sephadex G-15 chromatography. Fraction B4 was purified by electrophoresis at pH 6.5, when two major bands (B4a and B4b) were obtained. On electrophoresis at pH 1.9, material from band B4a gave one major (B4a1) and two minor bands (B4a2 and B4a3). Material from band B4b gave one major (B4b1) and one minor (B4b2) band. Amino acid compositions are shown in Table 1. Peptides from bands B3a, B4a1 and B4b1 were identical in amino acid composition, and the total yield was 112 nmol. The amino acid composition of the band-B4a1 material is shown in Table 1 as a typical example. These peptides presumably separated because of the homoserine/homoserine lactone equilibrium. Peptide B3b (72 nmol) was apparently the complete peptide sequence 2 (Fig. 2) that was not cleaved by CNBr (methionine and methionine sulphoxide were not detectable and presumably had been destroyed). Peptides B3c (3.7 nmol) B4a2 (2.1 nmol) B4a3 (1.3 nmol) and B4b2 (3.8 nmol) were minor incomplete-digestion products. These data show that it is Ser-5 on peptide sequence 2 (Fig. 2) that is concerned with pyruvate dehydrogenase inactivation. The data also suggest that it is primarily Ser-5 of peptide sequence 2 (Fig. 2) that is phosphorylated during addition of limiting quantities of ATP. These results agree with the results with bovine enzymes (Yeaman *et al.*, 1978).

That it is primarily Ser-5 that is phosphorylated during addition of limiting quantities of ATP to pyruvate dehydrogenase is supported also by experiments using the technique of electrophoresis at pH 1.9 to separate tryptic [^{32}P]phosphopeptides as described by Davis *et al.* (1977) and Yeaman *et al.* (1978). In these experiments, pyruvate dehydrogenase phosphate was not reduced and carbamoylmethylated, but was incubated with trypsin for up to 6 h. In their terminology, 91.0 ± 0.7% of ^{32}P was in site 1 of peptide T1, and 9.0 ± 0.6% of ^{32}P was in site 2 of peptide T2, i.e. 91% of ^{32}P was in Ser-5 of peptide sequence 2 and 9% was in Ser-12 of peptide sequence 2. Results are given as means ± S.E.M. for 12 observations. These results support our previous conclusions (Sugden & Randle, 1978).

Pyruvate dehydrogenase preparation C

The elution profile from QAE-Sephadex chromatography of a tryptic digest of CNBr-treated pyruvate dehydrogenase preparation C is shown in Fig. 1(*c*). Peak-C1 material was purified by electrophoresis at pH 6.5 and 1.9. Peak-C2 material gave three bands (C2a–C2c) at pH 6.5, components of which were further purified at pH 1.9. Peak-C3 material gave two peaks at pH 6.5 (C3a and C3b), material from which was further purified at pH 1.9. Peak-C4 material was electophoresed at pH 6.5 and 1.9, when it gave two bands (C4a and C4b). Peptide C1 (yield = 9.8 nmol) and C2a (13.0 nmol) corresponded to residues 4–9 of sequence 1 (Table 1 and Fig. 2). Peptide C2b (42.9 nmol) corresponded to residues 7–14 of sequence 2 (Table 1 and Fig. 2). Peptide C2c (30.0 nmol) was an incomplete-digestion product (results not shown). Peptide C3a (7.2 nmol) corresponded to residues 1–6 of sequence 2. Peptides C3b (4.3 nmol), C4a (13.5 nmol) and C4b (11.9 nmol) were incomplete-digestion products. These findings suggest that under the conditions used, phosphorylation was about twice as rapid at Ser-12 of sequence 2 as at Ser-6 of sequence 1. The finding that, for peptide C3a, the P content and amino acid analysis did not agree (Table 1) indicates that this site was mainly occupied by unlabelled P before incubation with [γ-^{32}P]ATP. About 14% was occupied by ^{32}P (on the basis of ^{32}P/glycine ratio), which agrees closely with the 10% of the enzyme remaining active after treatment with limiting quantities of ATP (see the Experimental section). Conversely, the closer agreement between P content and amino acid analysis for peptides C1 and C2b indicates that these sites are not phosphorylated by treatment with limiting quantities of ATP, and also confirms that it is Ser-5 of sequence 2 that is primarily concerned with pyruvate dehydrogenase inactivation.

Pyruvate dehydrogenase phosphate preparation D

This preparation (not reduced or carbamoylmethylated) was digested with trypsin for up to 6 h. The tryptic [^{32}P]phosphopeptides were separated by the technique of Davis *et al.* (1977) and Yeaman *et al.* (1978). After electrophoresis at pH 1.9 it was found that 84.0 ± 1.1% of P was in Ser-5 of peptide sequence 2 (Fig. 2), 15.0 ± 0.6% was in Ser-12 of peptide sequence 2, and about 1% was in Ser-6 of peptide sequence 1. Results are presented as means ± S.E.M. for 12 observations. They are significantly different from analogous observations for preparation B at $P < 0.001$. These results show that phosphorylation at Ser-5 (peptide 2) proceeds most rapidly, then phosphorylation at Ser-12 (peptide 2), with phosphorylation at Ser-6 (peptide 1) being least rapid.

The factors that determine this order of phosphorylation are not known. Since the purified peptide sequence 2 is a substrate for pyruvate dehydrogenase kinase with probably a greater specificity exhibited for Ser-5 than for Ser-12 (Davis *et al.*, 1977), the peptide itself must contain features enabling it to act as substrate. It is not known whether the isolated peptide contains any secondary structure or whether the determinants of phosphorylation reside exclusively in the primary sequence under these conditions. The use of the technique of Davis *et al.* (1977) and Yeaman *et al.* (1978) to separate the phosphopeptides from the pig heart enzyme is valid in spite of the apparent species difference at residue 8 in peptide 2 (Fig. 2) between pig heart and bovine kidney enzymes (aspartic acid and asparagine respectively), since at pH 1.9 neither residue is charged.

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