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Dephosphorylation of pig heart pyruvate dehydrogenase phosphate complexes by pig heart pyruvate dehydrogenase phosphate phosphatase

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1. Pig heart pyruvate dehydrogenase phosphate complex in which all three sites of phosphorylation were completely phosphorylated was re-activated at a slower rate by phosphatase than complex predominantly phosphorylated in site 1. The ratio of initial rates of re-activation was approx. 1:5 with a comparatively crude preparation of phosphatase and with phosphatase purified by gel filtration and ion-exchange chromatography. 2. The ratio of apparent first-order rate constants during dephosphorylation of fully phosphorylated complex averaged 1/3.8/1.3 for site 1/site 2/site 3. Only site-1 dephosphorylation was linearly correlated with re-activation of the complex throughout dephosphorylation. Dephosphorylation of site 3 was linearly correlated with re-activation after an initial burst of dephosphorylation. 3. Because dephosphorylation of site 1 was always associated with dephosphorylation of site 2, it is concluded that dephosphorylation cannot be purely random. 4. The ratio of apparent first-order rate constants for dephosphorylation of site 1 (partially/fully phosphorylated complexes) averaged 1.72. This ratio is smaller than the ratio of approx. 5 for the initial rates of re-activation. Possible mechanisms involved in the diminished rate of re-activation of fully phosphorylated complex are discussed.

Mammalian pyruvate dehydrogenase complexes (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3) are phosphorylated and inactivated (with MgATP) by a kinase intrinsic to the complex (Linn *et al.*, 1969). Fully phosphorylated bovine kidney and pig heart complexes contain three serine phosphorylation sites in α-chains of the decarboxylase component (EC 1.2.4.1) (Yeaman *et al.*, 1978; Sugden & Randle, 1978; Sugden *et al.*, 1979). During phosphorylation, inactivation is correlated with phosphorylation of one serine residue (site 1). Phosphorylation of the other two sites is largely accomplished after inactivation is complete. Relative rates of phosphorylation in the pig heart complex are approx. 15:3:1 (site 1/site 2/site 3) (Kerbey *et al.*, 1979). Because of this difference in rates, it is possible to prepare complexes that are either preferentially phosphorylated in site 1 (partially phosphorylated complex) or equally phosphorylated in all three sites (fully phosphorylated complex).

Dephosphorylation and re-activation of phosphorylated complexes is catalysed by a mitochondrial phosphatase extrinsic to the complex (Linn *et al.*, 1969). The initial rate of activation of partially phosphorylated complexes by phosphatase is approx. five times greater than that of fully phosphorylated complexes (Sugden *et al.*, 1978; Kerbey & Randle, 1979; Sugden & Simister, 1980). This suggested that the function of phosphorylation of sites 2 and 3 is to delay re-activation by phosphatase. Therefore in the present study we have investigated rates of dephosphorylation of individual sites in fully and partially phosphorylated complexes during re-activation by phosphatase. We have used a comparatively crude preparation of phosphatase prepared from pig heart muscle as in the studies of Sugden *et al.* (1978) and more highly purified phosphatase prepared from pig heart mitochondria, because preparations from heart muscle may contain extramitochondrial phosphatase(s) that can also dephosphorylate phosphorylated complexes (Stansbie *et al.*, 1976; Kerbey *et al.*, 1976).

Abbreviations used: SDS, sodium dodecyl sulphate; QAE-Sephadex, quaternary aminoethyl-Sephadex; h.p.l.c., high-pressure liquid chromatography; Mops, 4-morpholinepropanesulphonic acid.

Experimental

Materials

DEAE-cellulose (DE-52) was from Whatman

Ltd., Maidstone, Kent, U.K. Sources of other materials were as given by Sugden *et al.* (1979). Pyruvate dehydrogenase complex was purified from pig hearts (Kerbey *et al.*, 1979).

Methods and procedure

Assays. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Pyruvate dehydrogenase complex (direct asay), pyruvate dehydrogenase phosphate complex, $^{32}P_i$ release from pyruvate dehydrogenase [^{32}P]phosphate, and protein-bound [^{32}P]phosphate were assayed by methods given in Sugden *et al.* (1978, 1979) and Kerbey & Randle (1979). The distribution of ^{32}P between the three sites of phosphorylation in pyruvate dehydrogenase [^{32}P]phosphate was measured as described by Kerbey *et al.* (1979). Pyruvate dehydrogenase phosphate phosphatase was assayed during purification by the release of [^{32}P]P_i from fully phosphorylated complex after 4min of incubation at 30°C. For enzyme assays, 1 unit forms 1μmol of product/min at 30°C. SDS/polyacrylamide-gel electrophoresis was performed as described by Sugden & Randle (1978).

Pyruvate dehydrogenase phosphate phosphatase. Phosphatase preparation X was isolated from frozen pig hearts as described by Severson *et al.* (1974). More highly purified preparations of phosphatase were prepared from fresh hearts at 2°C as follows.

An extract of pig heart mitochondria containing pyruvate dehydrogenase complex (active and inactive forms) and phosphatase was prepared from 20 hearts as described by Kerbey *et al.* (1979). Phosphatase and complex were co-precipitated at pH7.0 with $(NH_4)_2SO_4$ (209g/litre), redissolved in the minimum volume of 20mM-potassium phosphate/0.5mM-EDTA/0.2mM-dithiothreitol, pH7.0, and dialysed for 60h against 2 × 50vol. of the same buffer. The dialysed material was adjusted to pH6.25 with 10% (v/v) acetic acid, insoluble material was removed (38000*g*, 20min), the supernatant adjusted to pH6.9 with 5M-KOH and centrifuged for 2.5h at 150000*g* to remove pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes. Phosphatase was then precipitated with $(NH_4)_2SO_4$ (209g/litre) at pH7.0, redissolved in 4ml of 20mM-potassium phosphate/0.5mM-EDTA, pH7.0 (phosphate/EDTA), and chromatographed on a column (80cm × 2.6cm diam.) of Sephadex G-100. Phosphatase activity was eluted in a single peak close to the void volume (calibration with Blue Dextran 2000). After precipitation with $(NH_4)_2SO_4$ (209g/litre), phosphatase was dissolved in 5ml of phosphate/EDTA and chromatographed on a column (92cm × 2.6cm diam.) of Sephadex G-150. Phosphatase activity was eluted in a single peak (162–328ml). This pooled material, termed preparation Y, contained 77% of the activity in the 150000*g* supernatant.

Preparation Y was chromatographed on a column (3.8cm × 2.7cm diam.) of DEAE-cellulose; phosphatase was eluted with a linear gradient of 0.1–0.4M-KCl (total vol. 300ml). As shown in Fig. 1, two peaks of activity were eluted at 0.19M-KCl (peak tube 60; preparation 60Z) and 0.24M-KCl (peak tube 67; preparation 67Z); recovery in the two peaks was approx. 60%. Preparations 60Z and 67Z were used within 12h of elution as they were unstable at 0 or −10°C. Attempts to concentrate these fractions by precipitation with $(NH_4)_2SO_4$ or ultrafiltration resulted in inactivation. The specific activities (P_i release; units/g of protein) were 31 (60Z) and 24 (67Z); purification was approx. 300-fold (60Z) and 230-fold (67Z). SDS/polyacrylamide-gel electrophoresis of 67Z showed one major band (63% total; ≡ mol.wt. 100000) and three minor bands (≡ mol.wts. approx. 174000, 41000 and 23000). Preparation 60Z showed additional bands corresponding to approx. mol.wts. of 150000 and 84000. The molecular weight of purified pig heart pyruvate dehydrogenase phosphate phosphatase has been reported as approx. 100000 (Siess & Wieland, 1972).

Pyruvate dehydrogenase [^{32}P]*phosphate.* Partially and fully phosphorylated complexes were prepared with [γ-^{32}P]ATP as described by Kerbey *et al.* (1979). Incorporations of ^{32}P and distribution of ^{32}P between sites were as given by Kerbey & Randle (1979). Trichloroacetic acid-soluble ^{32}P was <0.01%

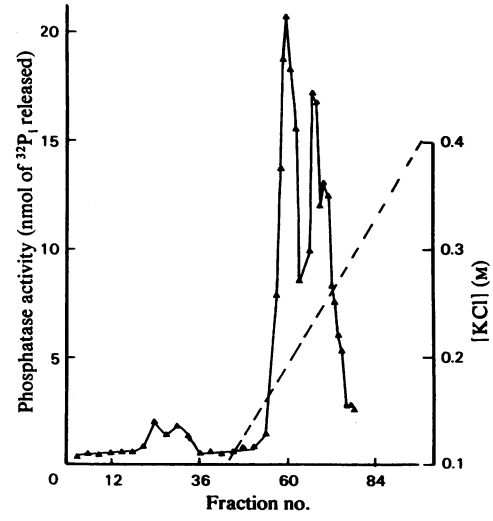

Fig. 1. *Chromatography of pig heart pyruvate dehydrogenase phosphate phosphatase on DEAE-cellulose*
For further details of methods used, see the Experimental section. ——, Phosphatase activity; ----, KCl concentration.

of total ^{32}P and increased to >98% after conversion with phosphatase. Pyruvate dehydrogenase complex activity (percentage of initial activity) was <6 (partial) or <2 (full). Recovery of active complex after conversion with phosphatase averaged 91%.

Studies of dephosphorylation and re-activation. These incubations were made at 30°C in 25 mM-Tris/HCl/10 mM-potassium phosphate/18 mM-$MgCl_2$ / 60 μM-$CaCl_2$ / 2 mM-dithiothreitol / pyruvate dehydrogenase phosphate (approx. 2.5 units · ml^{-1} as active complex), pH 7.0 (10 mM-Mg^{2+}, 43 μM-Ca^{2+}). The concentration of phosphatase was established empirically to give complete release of $[^{32}P]P_i$ in 20–40 min. With preparation X, ox serum (2%, v/v) was included as a proteinase inhibitor; no evidence of inactivation of pyruvate dehydrogenase complex after complete conversion was detected within 10 min (the longest period investigated). This concentration of Ca^{2+} is approx. $2 \times K_m$ for partially phosphorylated complex and approx. $0.5 \times K_m$ for fully phosphorylated complex; the concentration of Mg^{2+} was approx. $15 \times K_m$ for both preparations (Kerbey & Randle, 1979).

Thermolysin cleavage of tryptic phosphopeptides from pig heart pyruvate dehydrogenase [^{32}P]phosphate. Fully phosphorylated ^{32}P-labelled complex (80 units) was incubated with phosphatase for either 7.5 or 11.75 min at 30°C in 40 ml of 25 mM-Tris/HCl/10 mM-potassium phosphate/18 mM-$MgCl_2$/60 μM-$CaCl_2$/2 mM-dithiothreitol/2% (v/v) ox serum, pH 7.0. This resulted in release of either 37 or 49% of protein-bound ^{32}P as $[^{32}P]P_i$. The reaction was terminated by addition of trichloroacetic acid to 10% (w/v). Tryptic phosphopeptides were prepared and separated by high-voltage electrophoresis as described by Kerbey *et al.* (1979). Phosphopeptides A′ and A (for definition, see the Results and discussion section) were eluted with 0.1 M-NH_3, freeze-dried, taken up in 300 μl of 25 mM-HCl and chromatographed on a column (38 cm × 0.9 cm diam.) of Sephadex G-10. Fractions (0.55 ml) containing ^{32}P (Čerenkov radiation) were pooled, freeze-dried, and taken up in 1% (w/v) NH_4HCO_3. A portion of phosphopeptide was incubated for 3 h at 37°C with thermolysin (1.8 mg/ml), and the original and thermolysin-treated phosphopeptides were subjected to high-voltage paper electrophoresis at pH 1.9 on adjacent tracks of the same paper. The tracks were excised and 1 cm strips assayed for ^{32}P by liquid-scintillation spectrometry.

Amino acid composition of thermolysin-treated tryptic phosphopeptide A′. Partially phosphorylated complex (a mixture of 188 units of a preparation containing 0.57 nmol of P/unit of complex inactivated and 263 units of a preparation containing 0.43 nmol of P/unit) in 8 ml of 2% (w/v) NH_4HCO_3 was incubated for 1 h at 30°C with 9 mg of trypsin [90% of ^{32}P soluble in 10% (w/v) trichloroacetic acid] and then heated at 100°C for 5 min to inactivate trypsin. The bulk of the digest (8 ml) was incubated for 2 h at 37°C with 15 mg of thermolysin. High-voltage paper electrophoresis of original and thermolysin-treated phosphopeptide confirmed that complete cleavage of phosphopeptide A′, but none of phosphopeptide A, had occurred with thermolysin. The thermolysin cleavage product of tryptic phosphopeptide A′ was purified by (in succession): chromatography on Sephadex G-25 (Superfine grade; 45 cm × 2.6 cm diam. column) in 25 mM-HCl; high-voltage paper electrophoresis at pH 1.9 and pH 6.5 (see Sugden *et al.*, 1979); and chromatography on a column (1.1 cm × 1.5 cm diam.) of QAE-Sephadex A25 (see Sugden *et al.*, 1979). Desalting where necessary was accomplished by Sephadex G-15 chromatography in 25 mM-HCl. Acid hydrolysis and amino acid analysis showed that the phosphopeptide was not pure (amino acids not constituents of peptide A′ were present). Further purification by h.p.l.c. on Microbondapak C18 was achieved for us by Dr. J. Gagnon of the Department of Biochemistry, University of Oxford, Oxford, U.K. Successive runs in 0.1% (w/v) NH_4HCO_3 containing 5% (v/v) acetonitrile, in 0.1% NH_4HCO_3/2% acetonitrile and in 0.1% orthophosphoric acid/5% acetonitrile separated a ^{32}P-labelled peptide from other peptides. Amino acid analysis on a Durrum mark II amino acid analyser (Procedure 1) by Dr. J. Gagnon (see above) showed the composition $Asp_{0.7}$, $Ser_{1.3}$, $Gly_{2.0}$, $Met_{0.8}$, $Tyr_{1.0}$, $His_{1.9}$ and $Pro_{1.0}$, $^{32}P_{0.9}$ and trace amounts of Glu, Val and Lys (mean of duplicate runs). The expected recoveries for residues 1–10 of the phosphotetradecapeptide (see the Results and discussion section for amino acid sequence) were Asp_1, Ser_2, Gly_2, Met_1, Tyr_1, His_2, Pro_1, P_1. It is concluded that this phosphopeptide represents residues 1–10 of the tetradecapeptide phosphorylated in site 1. This conclusion assumes the partial destruction of serine that is known to occur during purification and/or acid hydrolysis (see Sugden *et al.*, 1979). Because of the considerable difficulties encountered in purification and consequential losses, there was insufficient material to correct for loss of serine by constructing a progress curve.

Results and discussion

Experiments with phosphatase preparation X

Rates of re-activation and $[^{32}P]P_i$ release. With phosphatase preparation X the initial rate of re-activation of partially phosphorylated complex was approximately five times that of fully phosphorylated complex; both yielded 2.8 units of active complex/ml after complete dephosphorylation (results not shown). The relationship between release of $[^{32}P]P_i$ and formation of active complex is shown in

Fig. 2. For partially phosphorylated complex this relationship was apparently linear over the range studied (0–70% re-activation). With fully phosphorylated complex, an apparently linear relationship was only seen after approximately two-thirds of protein-bound phosphate had been removed. The initial rate of $[^{32}P]P_i$ released/unit of active complex formed was approximately eighteen times greater for fully phosphorylated complex (PDHP 80/1) than for partially phosphorylated complex (PDHP 80/2). The findings of Sugden *et al.* (1978) are thus confirmed.

Dephosphorylation of individual sites in fully phosphorylated complex. The three sites of phosphorylation in pig heart complex are recovered in two tryptic phosphopeptides whose amino acid sequences (Sugden *et al.*, 1979) are shown below:

Site 1 Site 2

Tyr-His-Gly-His-Ser-Met-Ser-Asp-Pro-Gly-Val-Ser-Tyr-Arg

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Site 3

Tyr-Gly-Met-Gly-Thr-Ser-Val-Glu-Arg

1 2 3 4 5 6 7 8 9

During phosphorylation of pig heart complex with [γ-^{32}P]ATP, three ^{32}P-labelled phosphopeptides are separated by high-voltage electrophoresis at pH2 (Davis *et al.*, 1977; Kerbey *et al.*, 1979). These are termed A (tetradecapeptide phosphorylated in sites 1 and 2); B (nanopeptide phosphorylated in site 3); and A′ (tetradecapeptide phosphorylated in one site only) (Kerbey *et al.*, 1979). During phosphorylation of complex, only peptide A′ phosphorylated at site 1 was detected; the alternative form of A′ phosphorylated in site 2 alone was not detected (Yeaman *et al.*, 1978; Sugden *et al.*, 1979). This does not, however, preclude its occurrence during dephosphorylation (see below).

Fig. 3 shows the concentrations of phosphopeptides A′, A and B during dephosphorylation of fully phosphorylated complex (PDHP 80/1) with phosphatase preparation X. During dephosphorylation the concentration of phosphopeptide A (sites 1 and 2) fell rapidly and was replaced by A′ (site 1 or site 2 only). The concentration of B (site 3) showed an initial rapid decline, followed by an apparently linear decline in parallel with that of A′. The critical importance of the structure of phosphopeptide A′ in interpreting these data (i.e. whether all ^{32}P in A′ is in site 1) is illustrated in Fig. 4. This Figure shows that the rate of release of ^{32}P from site 1 + site 2 is approximately twice that of release from site 3. This is consistent with one of two alternative models, depending on the structure of A′. If A′ is a mixture of equal amounts of its two forms, then dephosphorylation is random and symmetrical (i.e. equal rates for all three sites). If A′ is wholly phosphorylated in site 1, then site 2 is dephosphorylated much more rapidly than site 1 or site 3 and dephosphorylation cannot be purely random, because dephosphorylation of site 1 does not occur without dephosphorylation of site 2. It was therefore important to locate the ^{32}P in A′ unambiguously.

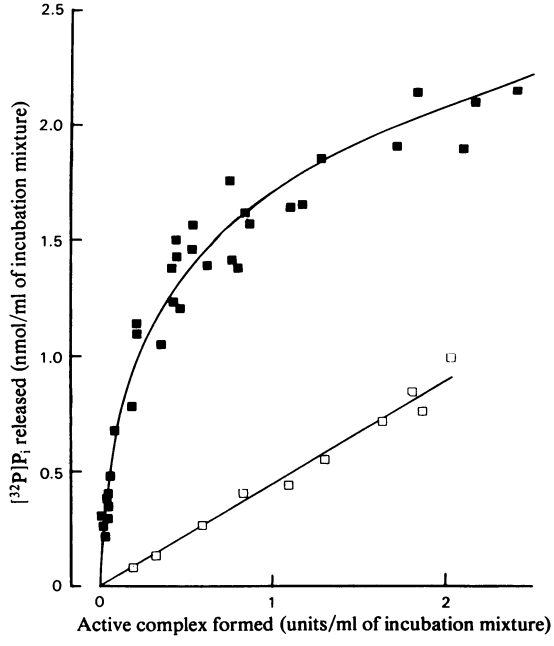

Fig. 2. *Release of* $[^{32}P]P_i$ *from partially* (□) *and fully* (■) *phosphorylated pig heart pyruvate dehydrogenase complexes by pig heart phosphatase (preparation X)*

For details of incubations and assays, see the Experimental section. At intervals, samples were taken for assay of active complex and the reaction was terminated by addition of trichloroacetic acid to 10% (v/v). $[^{32}P]P_i$ was assayed in the supernatant by liquid-scintillation spectrometry.

A number of possible methods for ascertaining the location of ^{32}P in phosphopeptide A′ were investigated. These include use of CNBr (cleavage between residues 6 and 7), staphylococcal proteinase (cleavage between residues 8 and 9), prolidase (cleavage between residues 9 and 10), thermolysin (cleavage between residues 10 and 11) and carboxypeptidase (sequential cleavage from residue 14). An essential requirement of this analysis is that

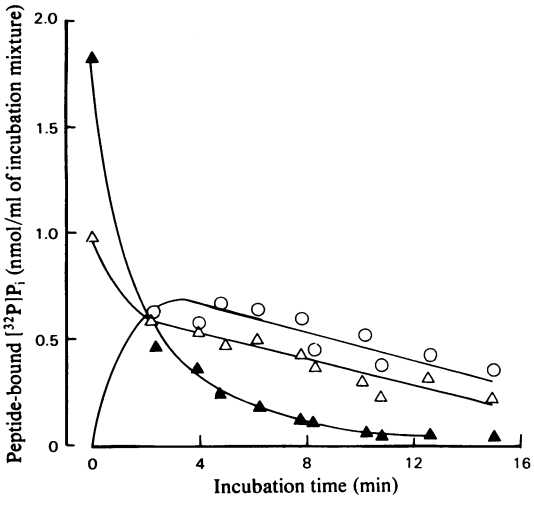

Fig. 3. *Tryptic ^{32}P-labelled phosphopeptides derived from fully phosphorylated pig heart pyruvate dehydrogenase [^{32}P]phosphate complex during hydrolysis with phosphatase preparation X*

For details of methods used, see the Experimental section. ▲, Phosphopeptide A (tetradecapeptide with sites 1 and 2 phosphorylated); O, phosphopeptide A′ (peptide A with only one site phosphorylated; △, phosphopeptide B (nanopeptide with site 3 phosphorylated). Points for each line are based on a total of 31 observations.

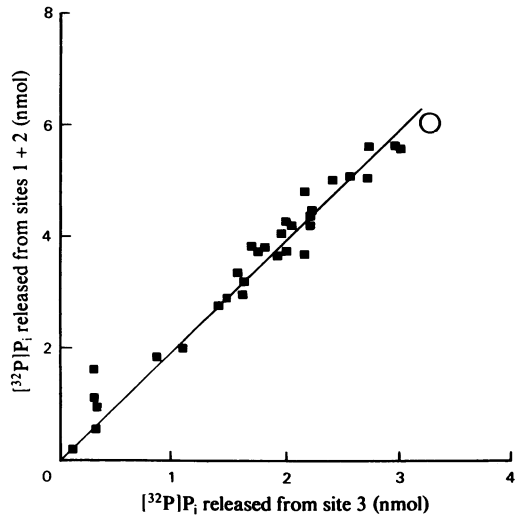

Fig. 4. *Correlation between [^{32}P]P_i released from sites 1 + 2 and site 3 of fully phosphorylated pig heart pyruvate dehydrogenase [^{32}P]phosphate with phosphatase preparation X*

The plot (■) is based on data shown in summary in Fig. 3. By least-squares linear-regression analysis the slope was 1.75 ± 0.09 (mean ± S.E.M.) for sites 1 + 2/site 3, r was 0.98 and the intercept 0.50 ± 0.28: the ratio of sites 1 + 2/site 3 in the original preparation of pyruvate dehydrogenase phosphate was 1.86 (O).

cleavage must be quantitative. Although cleavage was achieved with all reagents except prolidase, quantitative cleavage was achieved only with thermolysin. Thermolysin has been used previously to obtain evidence for absence of [^{32}P]peptide A′ phosphorylated in site 2 during phosphorylation of bovine kidney complex with [γ-^{32}P]ATP (Yeaman *et al.*, 1978).

The results of experiments in which [^{32}P]phosphopeptide A′ was cleaved with thermolysin are shown in Fig. 5. In this experiment, fully phosphorylated complex (PDHP 80/3) was dephosphorylated with phosphatase until 49% (Fig. 6) or 37% (result not shown) of protein-bound ^{32}P was released as [^{32}P]P_i. At this point, approx. 40% of protein-bound ^{32}P was recovered in phosphopeptide A′ (see Fig. 3). After trypsin treatment, phosphopeptide A′ was separated (from A and B) by high-voltage paper electrophoresis and a sample was incubated with thermolysin. As shown in Fig. 5, ^{32}P in phosphopeptide A′ was recovered quantitatively in a new phosphopeptide A″ with a lower electrophoretic mobility at pH 2 (identical findings after 37% dephosphorylation). Phosphopeptide A″ has also been generated with thermolysin by cleavage of phosphopeptide A′ derived by tryptic digestion of partially phosphorylated complex, purified, and its amino acid composition determined (see the Experimental section). The results of this analysis of phosphopeptide A″ indicated that it contained residues 1–10 of phosphopeptide A′ (i.e. that ^{32}P was in site 1). This leads us to conclude that phosphopeptide A′ formed by dephosphorylation of fully phosphorylated complex contains ^{32}P only in site 1.

It was therefore established that the occupancy of each of the sites of phosphorylation during dephosphorylation with phosphatase preparation X may be calculated from: site 1 (^{32}P in A′ + 0.5 of ^{32}P in A); site 2 (0.5 of ^{32}P in A); site 3 (^{32}P in B). The results of this analysis are shown in Fig. 6, which correlates release of [^{32}P]P_i from each site with the formation of active complex (PDHP 80/1). Fig. 6 shows that release of [^{32}P]P_i from sites 2 and 3 is not linearly correlated with the formation of active complex. Over 90% of site 2 was released before 20% of complex was activated; approx. 40% of site 3 was released during re-activation of 10% of complex. Site-3 release was biphasic, and release of the last 60% of site 3 was apparently linearly related to re-activation.

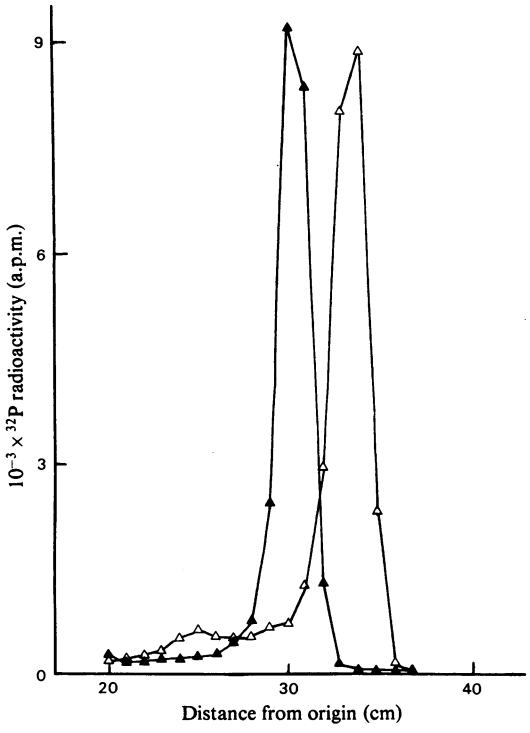

Fig. 5. *Effect of thermolysin on the electrophoretic mobility of tryptic phosphopeptide A' from pig heart pyruvate dehydrogenase [^{32}P]phosphate*

Fully phosphorylated pig heart pyruvate dehydrogenase [^{32}P]phosphate was 50% dephosphorylated by incubation at 30°C for 11.75 min with phosphatase as described in the legend to Fig. 2. The reaction was terminated with trichloroacetic acid. Tryptic phosphopeptide A' (for definition, see the legend to Fig. 3) was isolated by high-voltage paper electrophoresis and a portion was incubated at 37°C for 3 h with thermolysin (1.8 mg/ml). The original tryptic phosphopeptide, and the thermolysin-treated phosphopeptide, were subjected to high-voltage electrophoresis on adjacent tracks of the same paper, the tracks excised and 1 cm strips assayed for ^{32}P by liquid-scintillation spectrometry. △, Tryptic phosphopeptide; ▲, thermolysin-treated tryptic phosphopeptide. The recovery of ^{32}P in the phosphopeptide after thermolysin treatment was 100 ± 2% (mean ± S.E.M. for three experiments). For further details of methods used, see the Experimental section.

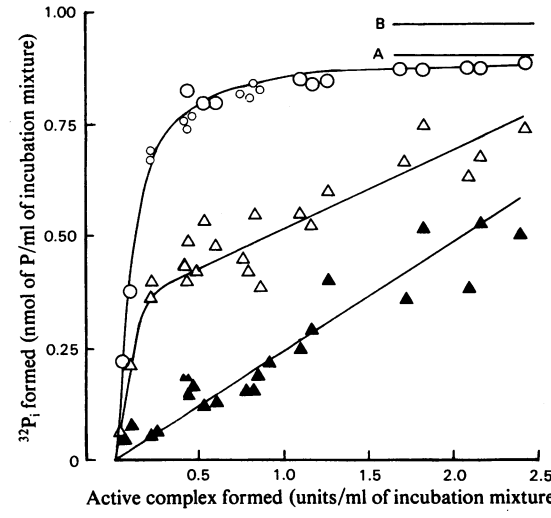

Fig. 6. *Correlation between active complex formed and [^{32}P]P_i released from sites 1, 2 and 3 of fully phosphorylated pig heart pyruvate dehydrogenase phosphate by phosphatase preparation X*

The conditions of incubation and assays of active complex, of [^{32}P]P_i release, and of ^{32}P in tryptic phosphopeptides are given in the Experimental section. Protein-bound ^{32}P by site was calculated from the product (total protein-bound ^{32}P) · (fraction of ^{32}P in each site). The release of [^{32}P]P_i from each site was then calculated as the difference from that present before incubation with phosphatase. ▲, ^{32}P released from site 1; ○, ^{32}P released from site 2; △, ^{32}P released from site 3. Line A, total ^{32}P in site 1 and in site 2 (i.e. site 1 = site 2); line B, total ^{32}P in site 3. For site 1, slope was 0.26 ± 0.02 nmol of P_i released/unit of active complex formed (mean ± S.E.M.); r was 0.95. The phosphorylated complex contained initially (in nmol of P/unit of complex inactivated), site 1, 0.37; site 2, 0.37; site 3, 0.38.

Release of [^{32}P]P_i from site 1 was apparently linearly related to the formation of active complex. Least-squares linear-regression analysis gave a correlation coefficient (r) of 0.95, an intercept of 0.13 ± 0.07 (mean ± S.E.M.; not significantly different from zero) and a slope of 0.26 ± 0.02 nmol of P_i released/unit of active complex formed (mean ± S.E.M.). The ratio of nmol of $^{32}P_i$ released from site 1/unit of active complex formed is 30% lower than the ratio of 0.37 nmol of ^{32}P incorporated/unit of complex inactivated in this fully phosphorylated complex. This apparent discrepancy may be consistent with the observed deviation from linearity in the relationship between ^{32}P incorporation into the complex and with inactivation during phosphorylation (see Sugden & Randle, 1978).

The apparent first-order rate constants for release of [^{32}P]P_i from sites 1, 2 and 3 of fully phosphorylated complex (PDHP 80/1) with phosphatase preparation X are shown in Table 1. The rate constant for site-2 dephosphorylation was approximately five times that for site 1 and three times that

Table 1. *Apparent first-order rate constants for the disappearance of [^{32}P]P_i from sites of phosphorylation in pig heart pyruvate dehydrogenase phosphate complexes incubated with pig heart phosphatase preparations*

The apparent first-order rate constants were calculated from least-squares linear-regression analysis of a plot of ln(protein-bound ^{32}P in sites in pyruvate dehydrogenase phosphate) against incubation time. The data for phosphatase preparation X were based on 23 observations with fully phosphorylated complex and 11 observations with partially phosphorylated complex. The data for phosphatase preparations 60Z and 67Z were based on six observations with partial or fully phosphorylated complexes. The conditions of incubation and methods of assay are given in the Experimental section. *$P < 0.01$ for difference from site 1 (partial); †$P < 0.01$ for difference from site 1 (full) and site 3 (full). Differences between site 1 (full) and site 3 (full) not significant ($P > 0.05$). PDHP is pyruvate dehydrogenase phosphate; individual preparations are shown by the number in parentheses.

Phosphatase preparation	PDHP complex	Apparent first-order rate constant (min^{-1}; mean ± S.E.M.) for dephosphorylation of: Site 1	Site 2	Site 3
X	Partial (80/2)	−0.117 ± 0.001	—	—
	Full (80/1)	−0.061 ± 0.005*	−0.28 ± 0.06†	−0.09 ± 0.01
60Z	Partial (80/5)	−0.044 ± 0.0007	—	—
	Full (80/6)	−0.031 ± 0.002*	−0.12 ± 0.01†	−0.039 ± 0.002
67Z	Partial (80/5)	−0.077 ± 0.001	—	—
	Full (80/6)	−0.042 ± 0.003*	−0.119 ± 0.004†	−0.051 ± 0.004

for site 3. The rate constant for site-3 dephosphorylation was 1.5 times that for site 1, but the difference was not statistically significant. The plots (not shown) were manifestly linear by eye for sites 1 and 2; that for site 3 showed an apparent initial rapid decline before linearity was achieved, but deviations from linearity were not significant. The data in Table 1 also show that the apparent first-order rate constant for dephosphorylation of site 1 in partially phosphorylated complex (PDHP 80/2) was significantly greater (1.92×; $P < 0.001$) than that for site 1 in fully phosphorylated complex (PDHP 80/1).

Experiments with phosphatase preparation Y

The initial rate of re-activation of partially phosphorylated complex (PDHP 80/5) was approximately seven times that of fully phosphorylated complex (PDHP 80/6) with phosphatase preparation Y (results not shown). Release of [^{32}P]P_i during re-activation was not investigated with phosphatase preparation Y.

Experiments with phosphatase preparations 60Z and 67Z

The initial rate of re-activation of partially phosphorylated complex (PDHP 80/5) was six times that of fully phosphorylated complex (PDHP 80/6) with phosphatase preparation 60Z (Fig. 7*a*) and 4.5 times with 67Z (Fig. 7*b*). With phosphatase preparation 60Z, the initial ratio of [^{32}P]P_i released/active complex formed with partially phosphorylated complex was eight times that with fully phosphorylated complex; with phosphatase preparation 67Z, the factor was approx. 3.5-fold (results not shown). With both 60Z and 67Z the rate of release of [^{32}P]P_i from site 2 was more rapid than the rate of release of [^{32}P]P_i from sites 1 and 3 (in fully phosphorylated complex). This is shown in Table 1, which gives the apparent first-order rate constants (that of site 2 approx. four times that of site 1; that of site 2 approx. 2.5 times that of site 3). As with phosphatase preparation X, the apparent first-order rate constant for site 3 was not significantly different from that for site 1. The initial more rapid dephosphorylation of site 3 was, however, apparent in the plot (result not shown). Table 1 also shows that the apparent first-order rate constant for dephosphorylation of site 1 in partially phosphorylated complex was significantly greater than that for dephosphorylation of site 1 in fully phosphorylated complex (1.42× for 60Z; 1.83× for 67Z).

General discussion and conclusions

In phosphate/Tris buffer the initial rate of re-activation of partially phosphorylated complex was approximately five times that of fully phosphorylated complex with comparatively crude preparations of phosphatase (Sugden *et al.*, 1978; Kerbey & Randle, 1979; the present study) or with more highly purified preparations of phosphatase from pig heart mitochondria (the present study).

In 50mM-Mops buffer (with 10mM-$MgCl_2$/100μM-$CaCl_2$), comparable rates of re-activation of partially and fully phosphorylated complexes by phosphatase are observed (Teague *et al.*, 1979; Kerbey & Randle, 1979; Sugden & Simister, 1980). It is therefore pertinent to consider the physiological role of site-2 and site-3 phosphorylations *in vivo*. Evidence published elsewhere indicates that site-2

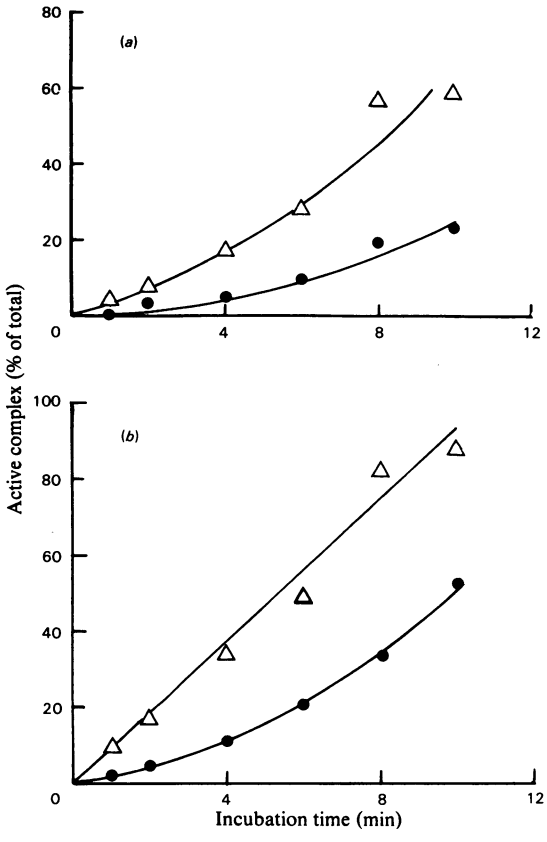

Fig. 7. *Rates of re-activation of partially phosphorylated* (△) *and fully phosphorylated* (▲) *pig heart pyruvate dehydrogenase phosphate complexes by phosphatase preparations 60Z* (*a*) *and 67Z* (*b*)

The details of incubation and assay were as in the legend to Fig. 2, except that incubation media contained either 38mM-KCl (60Z) or 48mM-KCl (67Z) added with phosphatase. The initial concentrations of phosphorylated complexes were; partial, 2.36 units/ml (0.45nmol of P/unit of complex inactivated); full, 2.57 units/ml (1.12nmol of P/unit of complex inactivated. Each point represents the mean result for two observations. For further details of the phosphatase preparations (from DEAE-cellulose chromatography), see Fig. 1 and the Experimental section.

and site-3 phosphorylations may inhibit re-activation by phosphatase in rat heart mitochondria and in rat heart *in vivo* in alloxan-diabetes and starvation (Sale & Randle, 1980, 1981; Hutson *et al.*, 1978). The suggestion (Teague *et al.*, 1979) that site-2 phosphorylation may be an alternative means of inactivation to site-1 phosphorylation lacks experimental support. During phosphorylation of active complex, phosphorylation of site 1 precedes that of site 2 (Yeaman *et al.*, 1978; Sugden *et al.*, 1979; Kerbey & Randle, 1979). During dephosphorylation of inactive complex, dephosphorylation of site 1 in the absence of dephosphorylation of site 2 has not been detected (by the analysis with thermolysin in the present study). Site-2 phosphorylation may only have a role in inactivation if it occurs in the absence of site-1 phosphorylation or if it enhances the inactivation induced by site-1 phosphorylation. There is currently no convincing evidence for either of these possibilities.

Release of P_i and of active complex from partially phosphorylated complex were pseudo-first-order reactions with identical rate constants. Release of P_i and of active complex from fully phosphorylated complex was more complicated. Release of P_i was not related linearly to release of active complex. Initially P_i release from fully phosphorylated complex was more rapid than P_i release from partially phosphorylated complex and was associated with a slower rate of formation of active complex. Only after substantial dephosphorylation (approx. 67%) was the relationship between release of P_i and of active complex comparable with that seen with partially phosphorylated complex. Further clarification was achieved by analysis of the rate of release of P_i from each of the three sites of phosphorylation. The high initial rate of release of P_i from fully phosphorylated complex was almost exclusively (approx. 95%) the result of dephosphorylation of sites 2 and 3 (2 > 3). Dephosphorylation of site 2 (and initially of site 3) was not correlated with formation of active complex. Dephosphorylation of individual sites conformed to the first-order rate equation (i.e. deviations from linearity in least-squares linear-regression analysis were not statistically significant). The order of apparent first-order rate constants was site 2 > site 3 > site 1. The difference between site 3 and site 1 was not significant. However, analysis by the first-order rate equation obscured the initially more rapid dephosphorylation of site 3, which was apparent in plots of the time course and was statistically significant (results not shown). The order of apparent first-order rate constants was qualitatively similar to that described by Teague *et al.* (1979), despite important differences in conditions of incubation detailed above.

In the present study the apparent first-order rate constant for dephosphorylation of site 1 in partially phosphorylated complex was approx. 1.7 times that for fully phosphorylated complex. Because site 1 is the inactivating site, this difference in the rate of dephosphorylation of site 1 is assumed to be an important factor in the different rates of re-activation of partially and fully phosphorylated complex. This conclusion receives some support from the observed correlation between dephosphorylation of site 1 and

re-activation of the complex in the present study. This conclusion may also be supported by the close correspondence of apparent first-order rate constants (site 1) under conditions where partially and fully phosphorylated complexes are dephosphorylated at comparable rates (Teague *et al.*, 1979). It is concluded that phosphorylation of sites 2 and 3 inhibits dephosphorylation of site 1 and re-activation of the complex under conditions used in the present study.

Dephosphorylation of the three sites in fully phosphorylated complex in the present study was not purely random. The studies with thermolysin failed to reveal evidence for dephosphorylation of site 1 independent of that of site 2; the rate of dephosphorylation of site 3 was biphasic. Teague *et al.* (1979) concluded that dephosphorylation of each of the three sites is purely random. However, their calculation of site occupancies assumed that dephosphorylation of site 1 does not occur independently of site 2. This has been established in the present study. Dephosphorylation of sites 1 and 2 is unlikely to proceed by an ordered mechanism. In complex phosphorylated in site 1 and thiophosphorylated in sites 2 and 3, phosphatase catalyses dephosphorylation of site 1 (Teague *et al.*, 1979; Radcliffe *et al.*, 1980). It is assumed that phosphatase does not catalyse dethiophosphorylation of sites 2 and 3. This may suggest that dephosphorylation of site 1 is linked to dephosphorylation of site 2.

The ratio of apparent first-order rate constants for dephosphorylation of site 1 (partial/full) was <2; the ratio of initial rates of re-activation (partial/full) was approx. 5. There are a number of possible reasons for this discrepancy, which cannot be resolved by current methods of analysis based on electrophoresis of tryptic phosphopeptides. This method is not sufficiently precise to provide accurate estimates of rates of dephosphorylation of individual sites at very early time points (such studies have been attempted, but errors were too great for unequivocal interpretation). Moreover, present methods of analysis do not allow computation of relative concentrations of decarboxylase units that are either fully dephosphorylated or fully phosphorylated or in the various possible states of partial dephosphorylation. These and other methods are required for a full elucidation of the mechanism whereby phosphorylation of sites 2 and 3 inhibits re-activation by phosphatase. The present study has shown unequivocally that phosphorylation of sites 2 and 3 inhibits re-activation with more highly purified phosphatase preparations from pig heart mitochondria and inhibits dephosphorylation of site 1; that dephosphorylation of site 1 in fully phosphorylated complex is not independent of that of site 2 and that the mechanism of dephosphorylation is not purely random.

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