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Pyruvate dehydrogenase kinase activity of pig heart pyruvate dehydrogenase (E1 component of pyruvate dehydrogenase complex)

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The pyruvate dehydrogenase (E1) and acetyltransferase (E2) components of pig heart and ox kidney pyruvate dehydrogenase (PDH) complex were separated and purified. The E1 component was phosphorylated (α-chain) and inactivated by MgATP. Phosphorylation was mainly confined to site 1. Addition of E2 accelerated phosphorylation of all three sites in E1α and inactivation of E1. On the basis of histone H1 phosphorylation, E2 is presumed to contain PDH kinase, which was removed (> 98%) by treatment with *p*-hydroxymercuriphenylsulphonate. Stimulation of ATP-dependent inactivation of E1 by E2 was independent of histone H1 kinase activity of E2. The effect of E2 is attributed to conformational change(s) induced in E1 and/or E1-associated PDH kinase. PDH kinase activity associated with E1 could not be separated from it be gel filtration or DEAE-cellulose chromatography. Subunits of PDH kinase were not detected on sodium dodecyl sulphate/polyacrylamide gels of E1 or E2, presumably because of low concentration. The activity of pig heart PDH complex was increased by E2, but not by E1, indicating that E2 is rate-limiting in the holocomplex reaction. ATP-dependent inactivation of PDH complex was accelerated by E1 or by phosphorylated E1 plus associated PDH kinase, but not by E2 plus presumed PDH kinase. It is suggested that a substantial proportion of PDH kinase may accompnay E1 when PDH complex is dissociated into its component enzymes. The possibility that E1 may possess intrinsic PDH kinase activity is considered unlikely, but may not have been fully excluded.

INTRODUCTION

The pyruvate dehydrogenase (PDH) complex of animal tissues consists of multiple copies of three component enzymes: E1 (pyruvate dehydrogenase, EC 1.2.4.1), E2 (dihydrolipoamide acetyltransferase, EC 2.3.1.12) and E3 (lipoyl dehydrogenase, EC 1.8.1.4). The complex also contains PDH kinase (EC 2.7.1.99), which with MgATP phosphorylates and inactivates pyruvate dehydrogenase. There are three sites of phosphorylation, only one of which (site 1) is a major inactivating site. Dephosphorylation and re-activation is accomplished by mitochondrial PDH phosphatase (EC 3.1.3.43) (for reviews see Reed, 1974, 1981; see also Stepp *et al.*, 1983; Teague *et al.*, 1982). Pettit & Reed (1982*b*) and Stepp *et al.* (1983) have described procedures to resolve the component enzymes of ox kidney and heart complexes by gel filtration at pH 9. PDH kinase co-purified with E2 and could be dissociated from it with *p*-hydroxymercuriphenylsulphonate.

We have attempted to use this procedure for resolution in order to obtain pig heart PDH complex free of PDH kinase (i.e. by recombination of E1 and E3 with E2 freed of PDH kinase). Starvation or alloxan-diabetes in rats results in phosphorylation and inactivation of PDH complex by stable mechanisms which increase the activity of PDH kinase (Hutson & Randle, 1978; Baxter & Coore, 1978; Kerbey & Randle, 1981, 1982; Kerbey *et al.*, 1984). We wished to use PDH complex free of PDH kinase as the basis of an assay for PDH kinase. However, in all of our experiments the E1 component derived from pig heart complex was phosphorylated and inactivated by ATP and is presumed to contain PDH kinase, which could not be separated from it by gel filtration or ion-exchange chromatography. In describing the discovery of reversible phosphorylation in the PDH complex, Linn *et al.* (1969) gave preliminary evidence that PDH kinase is an integral component of PDH (i.e. E1). Subsequent studies have led them to conclude that PDH kinase is an integral part of an E2–kinase sub-complex (e.g. see Linn *et al.*, 1972; Pettit & Reed, 1982*b*; Stepp *et al.*, 1983). In view of the complete consistency of our own findings, it seemed appropriate to report these and related observations.

EXPERIMENTAL

Materials

PDH complex was purified from pig hearts (or extracts of ox kidney mitochondria; Fatania *et al.*, 1981) by the method of Kerbey *et al.* (1979). The specific activity was 7–9 units/mg of protein, and the activity of 2-oxoglutarate dehydrogenase complex in such preparations was < 1% of the PDH complex activity. In some experiments complex was purified from pig heart mitochondrial extracts in the presence of proteinase inhibitors (1 mM-benzamidine/1 mM-phenylmethanesulphonyl fluoride). Lipoyl dehydrogenase was from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Sepharose 6B, Sephadex G-100 and Sephacryl S-300 were from Pharmacia (G.B.) Ltd., Hounslow, Middx., U.K. Histone H1 (type VS) was from Sigma Chemical Co.,

Abbreviations used: PDH, pyruvate dehydrogenase; SDS, sodium dodecyl sulphate.

Poole, Dorset, U.K. Sources of other materials were as in Kerbey & Randle (1981, 1982) and Kerbey *et al.* (1984).

Methods

Assays. PDH complex was assayed spectrophotometrically by measuring the rate of NADH formation (Cooper *et al.*, 1974) in the presence of lipoyl dehydrogenase (30 units/ml). E1 component was assayed as PDH complex by addition of an optimum amount of E2 (determined in pilot experiments) and of lipoyl dehydrogenase (30 units/ml). Protein was assayed by a biuret method (Gornall *et al.*, 1949). Incorporation of ^{32}P into PDH complex (native or reconstituted) or the E1 component and into individual phosphorylation sites was assayed by methods given in Kerbey *et al.* (1979) and Kerbey & Randle (1981). SDS/polyacrylamide-gel electrophoresis was carried out as described by Lau *et al.* (1981) except that 15 cm × 14 cm gels were used, and autoradiography as described by Kerbey *et al.* (1979). After staining and destaining (Coomassie Blue), gels were photographed and the photographs scanned by reflectance (Chromoscan 3; Joyce–Loebl). Autoradiographs were scanned by reflectance on a white background.

Resolution of component enzymes of PDH complex. PDH complex (70–300 units/ml: 275–3000 units) was resolved by gel filtration on Sepharose 6B (bed volume 265 ml) in 1 M-NaCl/0.1 M-glycine/1 mM-$MgCl_2$/0.1 mM-EDTA, pH 9, as described by Pettit & Reed (1982*b*). Elution volumes were 90–115 ml (E2 component) and 195–220 ml (E1 + E3 components) (by A_{280}). E2 was concentrated and E1 and E3 were separated and concentrated with $(NH_4)_2SO_4$ as described by Pettit & Reed (1982*b*). Preparations of E1 and E2 from PDH complex 84/2 and 84/3 were purified further by gel filtration on Sephacryl S-300 in 20 mM-potassium phosphate/2 mM-dithiothreitol/0.1 mM-EDTA, pH 7. Yields of E1 and E2 in these preparations were approx. 25% by protein and > 10% by activity (based on assay after recombination). Acetyltransferase (E2) was freed of PDH kinase by treatment with *p*-hydroxymercuriphenylsulphonate as described by Pettit & Reed (1982*b*). The procedure for resolution was varied in some experiments by preincubation of PDH complex with 10 mM-dithiothreitol for 4 h at 0 °C or by inclusion of 10 mM-mercaptoethanol or 1 mM-benzamidine during gel filtration; these treatments had no obvious effect.

ATP-dependent inactivation and phosphorylation. For measurement of phosphorylation and inactivation, PDH complex or E1 or (E1 + E2) were warmed to 30 °C (3 min) in 20 mM-potassium phosphate/2 mM-dithiothreitol/1 mM-$MgCl_2$, pH 7, and phosphorylation was initiated by addition of ATP (10 mM) to 0.5 mM final concn. (with [γ-^{32}P]ATP, sp. radioactivity was 150–300 d.p.m./pmol). Samples were taken for assay of activity (E1 or PDH complex), protein-bound ^{32}P and phosphorylation-site occupancies at times given in the Table or Figures. In each assay, control incubations showed no inactivation of PDH complex or of E1 in the absence of ATP. Control experiments also showed that ATP-dependent inactivation of E1 was not due to subsequent phosphorylation in the assay, which involved recombination of E1 with E2 and E3 in the presence of 0.2 μM-ATP (ATP transferred in the sample of E1). ATP-dependent inactivation of PDH complex or E1 was pseudo-first-order, and such PDH kinase activities are given as the apparent first-order rate constants computed as a Kerbey & Randle (1982).

RESULTS AND DISCUSSION

Subunit composition of preparations of pig heart pyruvate dehydrogenase complex and component enzymes

Unless stated otherwise, the studies described below were made with two preparations of pig heart PDH complex (84/2, 84/3) and with four preparations of acetyltransferase (E2) and pyruvate dehydrogenase (E1) (two from each preparation of complex). Results with 84/2 and 84/3 are generally representative of those obtained with six different preparations of pig heart complex and 11 preparations of E1 and E2.

Reflectance scans of photographs of representative SDS/polyacrylamide gels after electrophoresis in Tris buffer are shown in Fig. 1. PDH complex showed a fifth band (band 2 in Fig. 1*a*), in addition to the four usually resolved in phosphate gels (for review see Sugden & Randle, 1978). In ox heart complex this additional band has been observed by De Marcucci *et al.* (1985), and in Tris gels, but not in phosphate gels, by Stanley & Perham (1980). Its significance is not known, but a comparable band has been seen in purified preparations of lipoamide dehydrogenase from Boehringer (results not shown). Its presence in PDH complex was not prevented by the proteinase inhibitors benzamidine plus phenylmethanesulphonyl fluoride (results not shown). After resolution, E2 showed minor contamination with E3 [band 2 in Fig. 1(*b*); identified as E3 because PDH complex activity was seen after recombination with E1 devoid of E3 band(s)]. After resolution, E1 showed a minor band (2 in Fig. 1*c*), assumed to be derived from E1α, as it contained ^{32}P after phosphorylation of E1 with [γ-^{32}P]ATP (see below). Some preparations of E1 showed up to three further minor bands. One was E3, the second was band 2 of PDH complex (Fig. 1*a*), and the origin of the third (band 4 in Fig. 1*c*) is unknown. Gel filtration of E1 or of [^{32}P]phosphorylated E1 on Sephacryl S-300 (bed volume 170 ml) gave a single protein peak in which protein concentration and E1 activity or ^{32}P were closely correlated. The M_r was 136000, which approximates to the calculated M_r of 150000 for E1($\alpha_2\beta_2$) (with standards of M_r 670000, 158000, 44000, 17000, 1350; results not shown).

Assay of pyruvate dehydrogenase (E1) activity by reconstitution of PDH complex with acetyltransferase (E2) and lipoyl dehydrogenase (E3)

The main purpose of these experiments was to devise an assay for E1 activity based on the measurement of PDH complex activity after adding E1 to (E2 + E3). In pig heart PDH complex the molar ratio of E2/E1($\alpha_2\beta_2$) is 1 (Sugden & Randle, 1978). Preparations of E1 and E2 obtained by resolution of pig heart complex were completely devoid of PDH complex activity when assayed singly in the presence of saturating E3 (results not shown). In work described below, E2 concentrations are calculated on the basis of a subunit M_r of 52000 (see Pettit & Reed, 1982*a*).

When E2 (9 nmol/ml) was added to pig heart PDH complex (0.1 or 2 units/ml; approx. 0.06 or 1.2 nmol of E2 subunit/ml), the activity of PDH complex was increased in both experiments by 50 ± 0.63% (mean ±

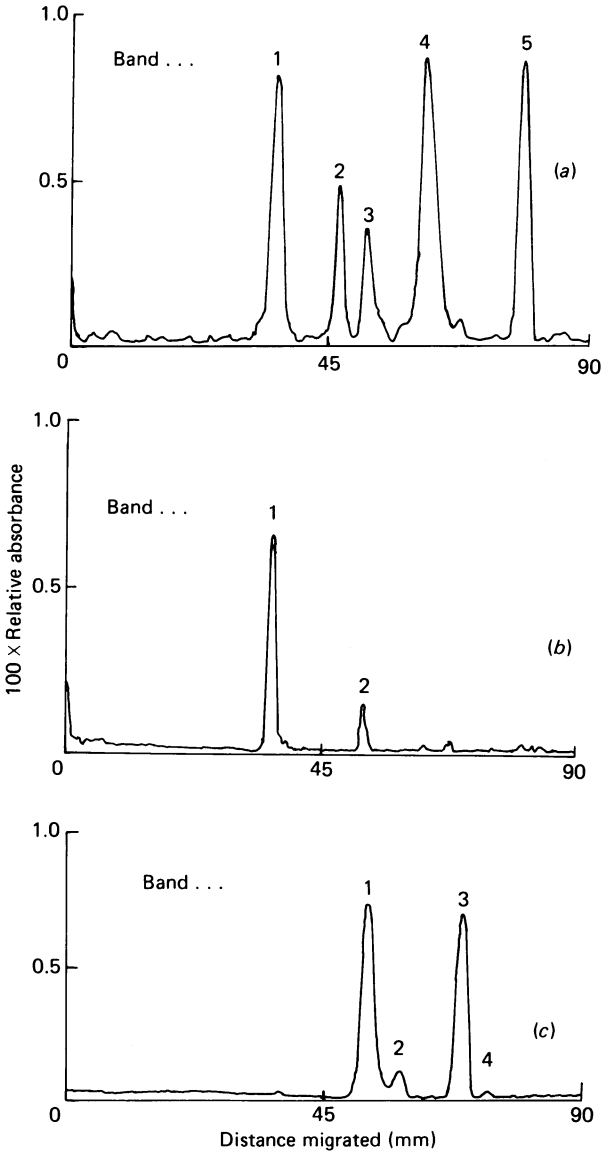

Fig. 1. Reflectance scans of photographs of SDS/polyacrylamide gels stained with Coomassie Blue

PDH complex preparation 84/3 was used. Bands are as follows (M_r; % of total). (*a*) 1, acetyltransferase subunit (77000; 21%); 2, unidentified (62000; 8%), 3, lipoyl dehydrogenase (56500; 7%); 4, pyruvate dehydrogenase, E1α (45000; 38%); 5, pyruvate dehydrogenase, E1β (33500; 26%). (*b*) Acetyltransferase: 1, acetyltransferase subunit (76500; 94%); 2, lipoyl dehydrogenase (57000; 6%). (*c*) Pyruvate dehydrogenase (E1): 1, E1α (43500; 51%); 2, degradation product of E1α (38300; 5%); 3, E1β (34000; 42%); 4, (?) degradation product of E1β (29000; 2%).

S.E.M. for eight observations, $P < 0.001$). No increase in activity was seen when E1 was added to PDH complex (results not shown). This indicated that activity of E2 is rate-limiting for the holocomplex reaction under the conditions of assay and suggested that a valid assay of E1 by reconstitution would be accomplished by use of a molar excess of E2 in the presence of a saturating concentration of E3.

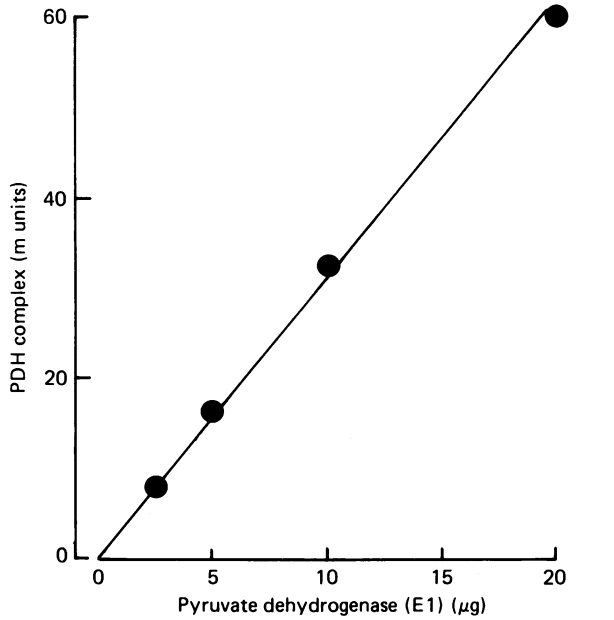

Fig. 2. Spectrophotometric assay of pyruvate dehydrogenase as pyruvate dehydrogenase complex formed by reconstitution with acetyltransferase and lipoyl dehydrogenase

Assay buffer contained, in addition to pyruvate and coenzymes (see Cooper *et al.*, 1974), acetyltransferase (16.5 μg; 318 pmol) and 40 units of lipoyl dehydrogenase (210 units/mg of protein). Reaction was initiated by addition of pyruvate dehydrogenase as shown, and activity was calculated from the intitial rate of NADH formation. Each point is the mean of five observations; 20 μg of E1 is approx. 130 pmol of E1($\alpha_2\beta_2$).

As shown in Fig. 2, a linear relationship between the amount of E1 added and PDH complex activity was achieved with molar ratios of E2/E1($\alpha_2\beta_2$) computed to be between 16:1 and 2:1. These assays were performed by adding E1 to a cuvette containing E2 and E3 and recording the initial rate of NADH formation. When E1 was preincubated with E2 at 30 °C before assay with E3, PDH complex activity increased to reach a plateau at an approx. 1.5-fold increase after 8 min (results not shown). In work described below, activities of E1 are given as maximum activities after preincubation, except for the following. In using the assay to measure E1 activity during phosphorylation of E1 with ATP, it was necessary to take the intitial rate on mixing (E1+E2+E3) in the cuvette, i.e. without preincubation. The values have been multiplied by 1.5 to correct for omission of preincubation. In studying phosphoryation of (E1+E2), the effect of ATP was ascertained from control incubations lacking ATP (i.e. to compensate for variable times of incubation of E1+E2).

The specific activity of PDH complex reconstituted, with preincubation, from 130 pmol of E1($\alpha_2\beta_2$) and 318 pmol of E2 with saturating E3 ranged from 1.9 to 2.7 units/mg of (E1+E2) for the preparations of E1 used in this study. These conditions correspond to the highest point in Fig. 2. This is approx. 30–40% of that of the original complexes. The specific activity at the molar ratio of E1/E2 pertaining to native complex (1:1) was not ascertained. It would have been greater than 1.9–2.7

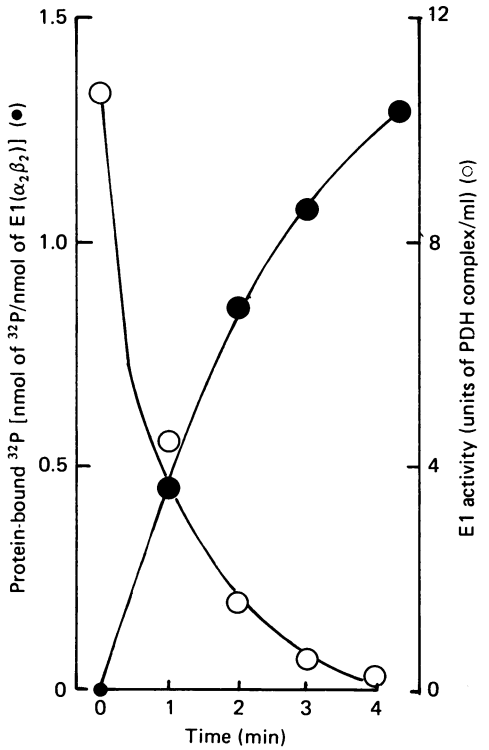

Fig. 3. ATP-dependent phosphorylation and inactivation of pyruvate dehydrogenase (E1)

Pyruvate dehydrogenase (E1) was incubated at 30 °C (4.05 mg/ml; 27 nmol of E1($\alpha_2\beta_2$)/ml) in 20 mM-potassium phosphate/2mM-dithiothreitol/0.1 mM-EDTA/1 mM-$MgCl_2$, pH 7.0, with 0.5 mM-[γ-^{32}P]ATP (170 d.p.m./pmol) and samples were taken for assay of activity [135 pmol of E1($\alpha_2\beta_2$)+384 pmol of acetyltransferase] and protein-bound ^{32}P at times shown. The apparent first-order rate constant for inactivation was 1.02 ± 0.03 min^{-1} (mean ± S.E.M.). Each point is the mean of five observations.

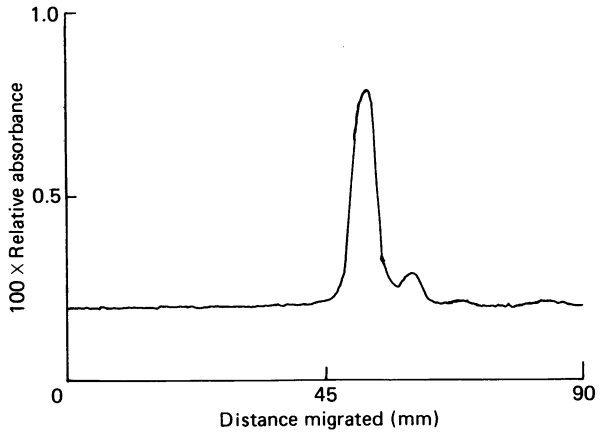

Fig. 4. Reflectance scan of autoradiograph of SDS/polyacrylamide-gel electrophoresis of [^{32}P]phosphorylated pyruvate dehydrogenase (E1)

The phosphorylated sample was prepared from fraction 58 after gel filtration of E1 on Sephacryl S-300 (see Fig. 6). The two radioactive bands correspond to bands 1 and 2 stained with Coomassie Blue in Fig. 1(*c*). From reflectance scanning, 94% of radioactivity was in the major peak and 6% in the minor peak.

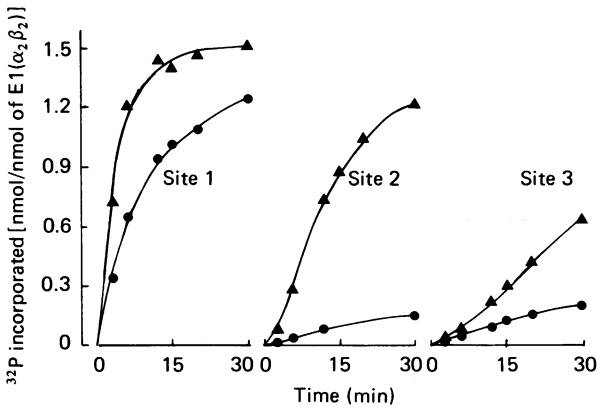

Fig. 5. Incorporation of ^{32}P from [γ-^{32}P]ATP into sites 1, 2 and 3 of pyruvate dehydrogenase (E1) in the absence (●) or presence (▲) of acetyltransferase (E2)

The general conditions of incubation were as in Fig. 3. Concentrations were: pyruvate dehydrogenase 0.5 mg/ml, 3.3 nmol of E1($\alpha_2\beta_2$)/ml; acetyltransferase 0.5 mg/ml, 9.6 nmol of E2 subunit/ml. Samples were taken at the times shown for total protein-bound ^{32}P and for analysis of site occupancy by tryptic digestion and high-voltage paper electrophoresis.

units/mg of (E1+E2), as the rate was not approaching a plateau at the highest point in Fig. 2.

Phosphorylation and inactivation of pyruvate dehydrogenase (E1) and the effect of acetyltransferase (E2)

Purified E1 component of PDH complex was rapidly phosphorylated and inactivated by [γ-^{32}P]ATP; inactivation was pseudo-first-order (Fig. 3). On SDS/polyacrylamide-gel electrophoresis, 93% of ^{32}P was associated with E1α and 7% with a band of lower M_r (Fig. 4; cf. Fig. 1*c*). The rate of inactivation was increased by the presence of E2 from 0.69 ± 0.02 to 0.95 ± 0.07 min^{-1} ($P < 0.01$ for difference between apparent first-order rate constants; mean ± S.E.M. for three experiments). Rates of inactivation of native PDH complex and of (E1+E2) did not differ significantly. The rate constants were 0.81 ± 0.03 and 0.77 ± 0.04 min^{-1} (means ± S.E.M. for three experiments, native complex first). The initial rate of phosphorylation of purified E1 component was 0.28 ± 0.06 nmol of ^{32}P/min per nmol of E1; this was increased by addition of E2. The ratio of initial rates [(E1+E2)/E1] was 2.72 ± 0.22 ($P < 0.001$ for difference from unity). These results for phosphorylation are means for a total of 12 preparations of E1 and E2 from six preparations of pig heart complex and two preparations of ox kidney complex. In recombination experiments the molar ratio of E2/E1($\alpha_2\beta_2$) was 1.36:1. Incorporation of ^{32}P from [γ-^{32}P]ATP into E2 alone was negligible and always < 5% of incorporation into E1 alone. In three of the phosphorylation experiments, rates of incorporation of ^{32}P into the three individual phosphorylation sites in the α-chain of E1 were measured. The results of one such experiment, which was typical of all three, are shown in Fig. 5. Because phosphorylation of site 1 was apparently

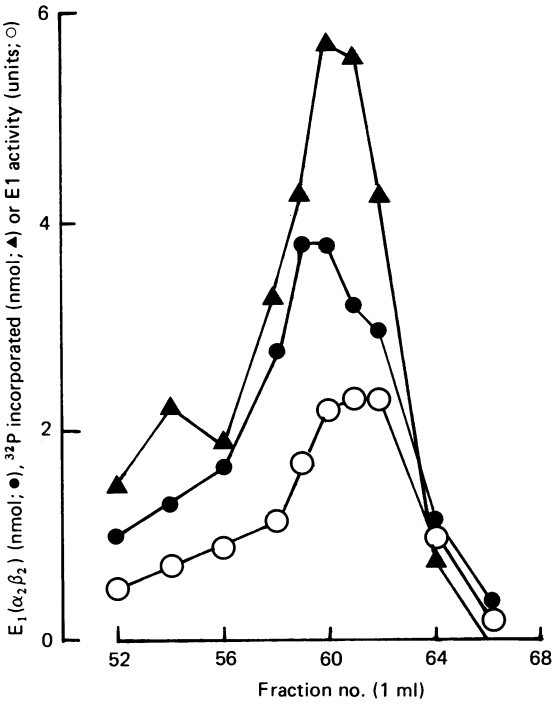

Fig. 6. Gel filtration of pyruvate dehydrogenase [4.88 mg of protein; 32.5 nmol of E1($\alpha_2\beta_2$)] on Sephacryl S-300

Column volume was 170 ml; void volume ('high-M_r protein aggregates') was 47 ml; elution volume of [^{32}P]P_i was 104 ml; 1 ml fractions were collected. Protein (●) was assayed by A_{280}, and nmol of E1 was calculated on the assumption of 100% recovery of protein. Activity of E1 (○) was assayed as PDH complex (318 pmol of E2; 7–76 pmol of E1). Incorporation of ^{32}P into E1 (▲) was measured after 30 min of incubation with [γ-^{32}P]ATP (200 d.p.m./pmol). Correlation coefficients (r) were 0.85, $P < 0.001$ (for units of activity against nmol of E1) and 0.94, $P < 0.001$ (for nmol of E1 against nmol of ^{32}P incorporated).

complete, it was possible to calculate rate constants by assuming equivalent incorporation at completion. The values (means ± S.E.M. for all three experiments, in min^{-1}; E1; E1 + E2; P for effect of E2) were: site 1 (0.065 ± 0.006; 0.3 ± 0.03; $P < 0.001$); site 2 (0.005 ± 0.0002; 0.035 ± 0.0005; $P < 0.001$); site 3 (0.005 ± 0.0002; 0.014 ± 0.0005; $P < 0.001$). Incorporation with E1 alone was confined mainly to site 1 (86%) (7% each in sites 2 and 3). Addition of E2 increased the rate of incorporation of ^{32}P into all three sites (Fig. 5); after 30 min 40% was in site 1, 37% in site 2 and 23% in site 3 (in native complex relative incorporations for sites 1, 2 and 3 were 39%, 34% and 27%; results not shown in Fig. 5). After 30 min of incubation the effect of E2 was confined largely to sites 2 and 3. Incorporations into site 1 were [nmol of ^{32}P/nmol of E1($\alpha_2\beta_2$)] 1.2 in the absence and 1.5 in the presence of E2. Both incorporations were < 1, suggesting some incorporation into the second α-chain; in PDH complex, incorporation was equivalent to phosphorylation of only one α-chain (Sugden & Randle, 1978). Total incorporation of ^{32}P into (E1 + E2) per unit of reconstituted complex inactivated, at completion, was approx. 3 times greater than with native PDH complex (results not shown). This is attributed to incorporation into the second α-chain (see above) and to lower recovery of activity on reconstitution.

Acetyltransferase (E2) is known to bind PDH kinase (Pettit & Reed, 1982*b*) and may have facilitated phosphorylation of E1 (the present work) by providing additional kinase or by inducing a more active conformation of kinase and/or E1 (cf. Pettit & Reed, 1982*b*). Treatment of E2 with *p*-hydroxymercuriphenylsulphonate decreased PDH kinase activity of E2 by > 98%, as assayed by a low rate of incorporation of ^{32}P from [γ-^{32}P]ATP into histone H1 (from 30 to 0.5 pmol/min; approx. 10 nmol of histone). Treatment of E2 with *p*-hydroxymercuriphenylsulphonate had no significant effect on E2 activity (assayed with E1 + E3) or on the rate constant for ATP-dependent inactivation of E1. The values (means ± S.E.M.; min^{-1}) were: untreated E2, 0.61 ± 0.03: *p*-hydroxymercuriphenylsulphonate-treated E2, 0.52 ± 0.09 (P for difference > 0.05). We conclude that E2 retained bioactivity after treatment with *p*-hydroxymercuriphenylsulphonate, that a substantial proportion of the PDH kinase in the PDH complex was associated with E1 after dissociation of the complex, and that the effect of E2 on phosphorylation of E1 may be due to a more active conformation of kinase and/or E1.

The question arises therefore as to whether we have purified an enzyme–substrate complex formed between PDH kinase and E1 or co-purified kinase and E1. After gel filtration on Sephacryl S-300 (Fig. 6), total protein, activity of E1 and the extent of total incorporation of ^{32}P from [γ-^{32}P]ATP into E1 over 30 min were closely correlated in all fractions of the peak of protein. From Sephadex G-100, E1 was eluted as a single peak close to the void volume, and the rate and extent of incorporation of ^{32}P from [γ-^{32}P]ATP into E1 were unchanged (results not shown). Ion-exchange chromatography on DEAE-cellulose (DE52) with a linear gradient of 0–0.5 M-KCl failed to separate E1 and kinase activities. Therefore by these criteria E1 and kinase activities are closely associated. PDH kinase is a dimer of an α-subunit (M_r 48000) and a β-subunit (M_r 45000), kinase activity being associated with the α-subunit (Stepp *et al.*, 1983). SDS/polyacrylamide-gel electrophoresis as used in the present study is known to separate E1α from proteins of M_r 46000 or greater (e.g. see Fatania *et al.*, 1981). No protein band corresponding to the α-subunit of PDH kinase has been seen in SDS/polyacrylamide-gel electrophoresis of E1 or E2 or PDH complex (Fig. 1; other results not shown), presumably because the concentration of PDH kinase is too low (Stepp *et al.*, 1983; Pettit & Reed, 1982*b*). Sephacryl S-300 chromatography as employed in the present study would be expected to separate E1($\alpha_2\beta_2$) and PDH kinase by three tubes (based on M_r values of 136000 and 93000 respectively, assuming comparable axial symmetry), and to separate E1($\alpha_2\beta_2$) and (E1–PDH kinase) by five tubes (based on M_r of 136000 and 229000). On this basis possible complexes that would not be expected to be separated might include [E1($\alpha\beta$) + PDH kinase($\alpha\beta$); computed M_r 168000] or [E1($\alpha\beta$) + PDH kinase(α); computed M_r 123000].

The function of the β-subunit of PDH kinase is not known, but it has been suggested that it is a regulatory subunit. Regulation of the PDH kinase reaction with purified preparations of E1 has not been systematically investigated, but incorporation of ^{32}P from [γ-^{32}P]ATP into E1 was inhibited by 70–90% by 5 mM-pyruvate during 1–10 min of incubation (results not shown).

Table 1. Effect of pyruvate dehydrogenase (E1) and of phosphorylated pyruvate dehydrogenase (E1 phosphate) on the activity of PDH complex and the rate of inactivation by ATP-dependent phosphorylation

Pig heart PDH complex (0.2 unit/ml)+lipoyl dehydrogenase (30 units/ml) were incubated at 30 °C in 20 mM-potassium phosphate/2 mM-dithiothreitol/1 mM-$MgCl_2$, pH 7, and phosphorylation was initiated by addition of ATP to 0.5 mM. Samples were taken for assay of PDH complex at 0, 0.5, 1, 1.5 and 2 min. Rate constants are means ± S.E.M. for three observations at each time point (15 observations in all). At each concentration $P < 0.01$ for effects of E1 on ATP-dependent inactivation of PDH complex, for effect of E1 phosphate to inactivate PDH complex and for effect of E1 phosphate on ATP-dependent inactivation of PDH complex. The concentration of E1 in PDH complex was computed to be 0.1 nmol of E1$\alpha_2\beta_2$/ml (assuming 10 units/mg of protein).

Addition	Apparent first-order rate constant (min^{-1}) at concentration [nmol of E1($\alpha_2\beta_2$)/ml]: 0	0.11	0.23	0.45
E1, ATP	0.15±0.02	0.33±0.04	0.34±0.03	0.50±0.04
E1 phosphate (*a*)	—	0.07±0.01	0.25±0.01	0.60±0.03
E1 phosphate, ATP (*b*)	0.15±0.02	0.98±0.03	1.21±0.05	1.20±0.05
(*b*)−(*a*)	0.15±0.02	0.91±0.03	0.96±0.05	0.60±0.06

Effect of component enzymes of the PDH complex on activities in nati:e PDH complex

Effect of E1 and E2 on PDH complex activity. Addition of E1 [110, 225 or 450 pmol of E1($\alpha_2\beta_2$)/ml] to PDH complex (200 munits/ml; equivalent to approx. 100 pmol of E1 or E2) had no effect on PDH complex activity (results not shown). Addition of E2 (9.6 nmol of E2 subunit/ml) to PDH complex (0.1 or 2 units/ml; equivalent to approx. 50 pmol or 1 nmol/ml of E1 or E2) increased PDH complex activity by 50.4% ($P < 0.05$ for increase) and 50.6% ($P < 0.001$ for increase) respectively. Apparently E2 is rate-limiting for the holocomplex reaction, at least under the conditions of assay. This finding may provide an explanation for the sigmoid relationship between phosphorylation of E1α and inactivation of PDH complex. A linear and maximum rate of inactivation of PDH complex by phosphorylation of the inactivating site was only observed after approximately one-third of the sites were phosphorylated (Sugden & Randle, 1978). Evidence that E1 is transferred from PDH complex to E2 has been given by Cate *et al.* (1980).

Effect of E1 and E2 on ATP-dependent inactivation of PDH complex. Addition of E2 to PDH complex (for concentrations see the preceding paragraph) had no effect on the rate of ATP-dependent inactivation of PDH complex. Apparent first-order rate constants (min^{-1}; means ± S.E.M. for three observations at 0, 0.5, 1, 1.5 and 2 min) were 0.24±0.03 in the absence and 0.25±0.06 in the presence of E2.

The effect of E1 or of phosphorylated E1 (E1 phosphate) on the rate of ATP-dependent inactivation of PDH complex is shown in Table 1. A low concentration of PDH complex was used (0.2 unit/ml), because this gives a low rate of ATP-dependent inactivation (Kerbey & Randle, 1982). As shown in line 1 of Table 1, E1 increased the rate of ATP-dependent inactivation of PDH complex 2–3-fold (depending on E1 concentration). This is assumed to be an effect of PDH kinase associated with E1, but interpretation of quantitative aspects of the stimultion is complicated by activation of phosphorylated complex by E1 (result not shown; Pratt *et al.*, 1979). As shown in line 2 of Table 1, E1 phosphate inactivated PDH complex in the absence of ATP. Two interpretations of this finding are possible. Exchange of E1 phosphate for E1 in PDH complex occurs, but such exchange is slow relative to exchange of E1 for E1 phosphate (Pratt *et al.*, 1979). Despite extensive dialysis, ATP could not be totally removed from E1 phosphate. By assay with ATP-dependent luciferase, the ATP concentration with 0.45 nmol of E1 phosphate/ml was approx. 5 μM ($0.25 \times K_m$ for PDH kinase reaction). The rate of inactivation of PDH complex at this concentration of E1 phosphate was 4-fold greater than that by 0.5 mM-ATP in the absence of E1 phosphate. As shown in lines 3 and 4 of Table 1, E1 phosphate increased the rate of ATP-dependent inactivation of PDH complex markedly (0.5 mM-ATP). The obvious interpretation of this finding is that PDH kinase associated with E1 or E1 phosphate increased the rate of phosphorylation of E1 in PDH complex. The possibility that E1 phosphate or E1 may stimulate the PDH kinase reaction in PDH complex could not be excluded, because we have been unable to obtain E1 free of PDH kinase.

General discussion and conclusions

Autophosphorylation of preparations of PDH (E1) was described by Linn *et al.* (1969). They suggested that PDH kinase was a subunit of E1 and that the effect of E2 to accelerate phosphorylation was the result of conformational change(s). Subsequently Linn *et al.* (1972) reported that PDH kinase was associated with E2 and commented that autophosphorylation of E1 could be decreased by crystallization of E1. PDH kinase was purified after dissociation from E2 (Stepp *et al.*, 1983).

In the present study all of 13 different preparations of purified E1 (11 pig heart; 2 ox kidney) have shown phosphorylation by ATP, and associated inactivation was shown with three of them (pig heart). Phosphorylation was largely confined to site 1. Phosphorylation and inactivation were accelerated by E2, which was essential for substantial phosphorylation of sites 2 and 3. The effect of E2 did not appear to depend on the presence of PDH kinase in E2, and the rate of ATP-dependent inactivation of E1 was apparently not influenced by the presence of PDH kinase in E2. This may indicate that the

predominant effect of E2 is exerted through conformational change(s).

The most likely explanation for autophosphorylation of E1 is the presence of PDH kinase in E1 as a result of tight binding. However, we have been unable to obtain positive evidence for the presence of PDH kinase in E1. Gel filtration or ion-exchange chromatography failed to separate E1 and kinase activity; subunits corresponding to PDH kinase were not seen on SDS/polyacrylamide-gel electrophoresis of E1; and the M_r of E1 and associated kinase activity on gel filtration did not correspond to a complex formed between ($E1(\alpha_2\beta_2)$ and PDH kinase. A distinct and specific PDH kinase has been purified by Stepp *et al.* (1983), and it seems unlikely that E1 possesses intrinsic kinase activity. However, evidence which may completely exclude this possibility is at present lacking. The question could presumably be resolved by immunoassay of PDH kinase subunits. For reasons that are not apparent, the association between E2 and PDH kinase appears to have been emphasized, and the kinase activity associated with E1 has received very little consideration. The present studies might suggest that it is quantitatively as important as PDH kinase associated with E2 and that there may be important and unresolved questions to be answered.

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