INACTIVATION OF PIG HEART PYRUVATE DEHYDROGENASE COMPLEX BY ADENOSINE-5'-*O*(3-THIOTRIPHOSPHATE)

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1. Introduction

Porcine and bovine PDH complexes are inactivated and phosphorylated with MgATP by a kinase intrinsic to the complex. Fully phosphorylated PDHP complex ($\alpha_2P_3\beta_2$) contains 3 phosphorylated serine residues. These are recovered after tryptic digestion in a tetradecapeptide (site 1, Ser 5; site 2, Ser 12) and a nonapeptide (site 3, Ser 6) of known amino acid sequence [1,2]. Discontinuous phosphorylation by incremental additions of a limiting amount of ATP leads to selective phosphorylation of site 1 yielding PDHPcomplex ($\alpha P \cdot \alpha\beta_2$) [1,3]. This and other methods [2,4] have shown that inactivation is correlated with phosphorylation of site 1. Phosphorylation of sites 2 and 3 inhibits reactivation of the complex by PDHP phosphatase [5,6]. Investigation of the physiological significance of site 2 and site 3 phosphorylations would be facilitated if one or more sites of phosphorylation could be blocked by groups which are resistant to attack by PDHP phosphatase. Conversion of phosphorylase *b* to phosphorylase *a* with phosphorylase kinase has been accomplished with ATPγS. The resultant (presumed) thiophosphoryl phosphorylase is resistant to *a*–*b* conversion by phosphorylase phosphatase [7]. We describe here inactivation of pig heart PDH complex by ATPγS, give evidence that this involves thiophosphorylation of the complex and that inactive PDH(SP) complex is resistant to reactivation by PDHP phosphatase.

Abbreviations: ATPγS, adenosine-5'-*O*(3-thiotriphosphate); DTT, dithiothreitol; EGTA, ethanedioxybis (ethylamine)-tetracetate; ($\alpha_2\beta_2$), tetrameric pyruvate decarboxylase (EC 1.2.4.1); PDH complex, pyruvate dehydrogenase complex; PDHP and PDH(SP) complexes, pyruvate dehydrogenase phosphate and thiophosphate complexes

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2. Experimental

2.1. *Materials*

ATPγS was from BCL, Lewes, Sussex. Manufacturers analysis showed 12% ADP; this was confirmed by ion-exchange thin-layer chromatography on PE1-cellulose [8] which also showed no more than a trace of ATP. Sources of other biochemicals, radiochemicals, PDH complex and ox heart PDHP phosphatase are given in [3,6,9].

PDHP complex ($\alpha P \cdot \alpha\beta_2$) was prepared by discontinuous phosphorylation [5] in 20 mM potassium phosphate/2 mM DTT/ 0.2 mM $MgCl_2$ (pH 7) with [γ-^{32}P]ATP (110 dpm/pmol). PDH(SP) complex was prepared by exactly the same method substituting ATPγS for ATP. The complexes were recovered by centrifugation through 4 vol. 2% (w/v) sucrose in phosphate/DTT [9] (90 min, 4°C, 150 000 × *g*) and taken up in phosphate/DTT; or dialysed for 60 h at 4°C against 4 changes of 100 vol. phosphate/DTT. Recovery of PDHP complex (based on protein-bound ^{32}P) was >95%; it is assumed that the recovery of PDH(SP) complex was the same. Based on this recovery preparations of PDHP and PDH(SP) complexes contained <5% of active complex. Incorporation of ^{32}P was 0.45–0.51 nmol/unit PDH complex inactivated.

For conversion to [$\alpha_2P \cdot (SP)_2\beta_2$], PDHP complex ($\alpha P \cdot \alpha\beta_2$) was incubated for 60–90 min at 30°C with 0.42–0.5 mM ATPγS/1 mM $MgCl_2$. Evidence for this conversion is given in section 3.1. During incubation

with ATPγS, loss of protein-bound ^{32}P (5% in 60 min) suggested that if exchange of protein-bound ^{32}P with the thiophosphoryl group of ATPγS occurs, it is slow. The thiophosphoryl PDHP complex was dialysed for 60 h at 4°C (4 × 100 vol. phosphate/DTT with 1 mM EDTA in the first 3). Conversion of PDH(SP) complex to $[\alpha_2(SP) \cdot (P)_2\beta_2]$ was effected by incubation for 60 min at 30°C with 0.5 mM [γ-^{32}P]ATP (176 dpm/pmol) and 1 mM $MgCl_2$. Incorporation of ^{32}P was 0.64 nmol/unit PDH complex thiophosphorylated.

The ATPase activity of PDH complexes hydrolysed <1% of ATP/min under conditions of fig.1.

2.2. *Methods*

PDH complex was assayed spectophotometrically by NADH formation [10] (1 unit is 1 μmol NADH formed/min). ATP and ATPγS were assayed spectrophotometrically in 1 M HCl at 257 nm. Protein-bound ^{32}P was assayed as in [5]. Incorporation of ^{32}P into sites 1–3 in PDHP complexes was assayed after tryptic digestion and high-voltage paper electrophoresis [3]. Incorporation of ^{14}C from [1-^{14}C]acetyl CoA into lipoyl residues in PDHP and PDH(SP) complexes was determined as in [9]. Acetyl CoA solutions were standardised spectrophotometrically [3]. $^{32}P_i$ was assayed in PDH ^{32}P complexes after trichloroacetic acid precipitation (10% w/v) in the presence of bovine serum albumin [10]. ATPase activity was measured by $^{32}P_i$ release from [γ-^{32}P]ATP [10].

Release of active PDH complex and of $^{32}P_i$ from $(\alpha P \cdot \alpha\beta_2)$ $[\alpha(SP)\alpha\beta_2]$ and $[\alpha_2P(SP)_2\beta_2]$ with PDHP phosphatase was assayed following incubation in 10 mM potassium phosphate/25 mM Tris–HCl/2 mM DTT/10 mM EGTA/9.75 mM $CaCl_2$/25 mM $MgCl_2$ (pH 7) (medium A).

3. Results and discussion

Indirect methods were necessary to demonstrate thiophosphorylation of PDH complex by ATPγS because it has not been possible to prepare [^{35}S]-ATPγS. We have prepared sodium thiophosphate, [^{35}S]thiophosphate and acetyl thiophosphate by established methods. The synthesis of sodium thiophosphate was confirmed by ^{31}P NMR which showed also that contamination with sodium phosphate was <2%. It has not been possible to prepare [^{35}S]ATPγS in sufficient yield or of utilisable specific activity by employing methods of exchange [11,12] or net synthesis based on triose phosphate dehydrogenase + diphosphoglycerate kinase or acetate kinase [13].

3.1. *Thiophosphorylation of pig heart PDH complex*

Figure 1 shows that pig heart PDH complex is inactivated by incubation with ATPγS. Panel (a) in fig.1 shows that the rate of inactivation with ATPγS is slower than with ATP at equivalent concentrations (0.5 mM). Apparent K_m values were (mean ± SEM) 6.26 ± 2.36 μM MgATP and 6.32 ± 1.67 μM MgATPγS (3 obs. each at 11, 24, 55 and 201 μM MgATP; and 9,21, 48 and 176 μM ATPγS). The values for V_{max} (mean ± SEM in % of complex inactivated in 1 min) were 50.4 ± 3.46% (ATP) and 34.6 ± 1.81% (ATPγS) (P for difference <0.001). The difference in rates of inactivation in fig.1(a) thus appears to be the result of differences in V_{max}. It should be noted that ATPγS contained 12% of ADP (a competitive inhibitor of the PDH kinase reaction) whereas ATP contained only 0.5% of ADP (manufacturers analysis). However the K_i for ADP is much higher than the K_m for MgATP and MgATPγS and the presence of 12% of ADP should only reduce the calculated V_{max} by 6% (assuming K_i for ADP with ATPγS is the same as with ATP). There is evidence that ATPγS can form

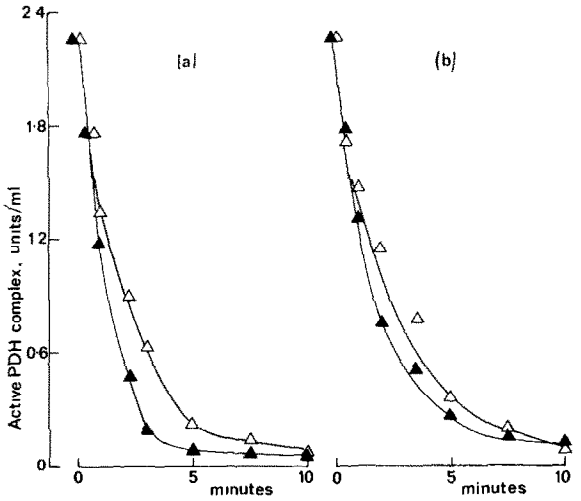

Fig.1. Incubations made at 30°C in (a) phosphate/DTT (1 mM $MgCl_2$) with 0.5 mM ATP (▲) or 0.5 mM ATPγS (△) and in (b) only, 1 mM NAD^+. After 5 min preincubation, reaction initiated with PDH complex (to 2.26 units/ml) and samples for assay of PDH complex taken at times shown. Points are means of 2 obs.

disulphides with protein-SH groups [14] and it seemed important to exclude the possibility of disulphide formation with the lipoyl moieties of the PDH complex and consequent inactivation. As shown in fig.1(b), NAD^+ (which oxidises lipoyl residues in the complex) had no effect on inactivation of PDH complex by ATPγS. Rates with ATP and with ATPγS were slower in the presence of NAD^+ as expected [3]. The integrity of lipoyl residues was shown directly by acetylation with [1-^{14}C]acetyl CoA in the presence of NADH. Incorporations (mean ± SEM for 6 obs. in nmol acetyl/unit PDH complex equiv.) were 0.77 ± 0.01 (PDHP complex) and 0.76 ± 0.01 [PDH(SP) complex]. It is known that phosphorylation of the PDH complex does not inhibit the dihydrolipoate acetyltransferase or lipoyldehydrogenase reactions [9]; our findings show that thiophosphorylation does not inhibit these reactions.

Indirect evidence for thiophosphorylation of each of the 3 sites of phosphorylation in the PDH complex may be summarised as follows. PDH^{32}P complex ($\alpha P \cdot \alpha\beta_2$) prepared by the discontinuous method of phosphorylation shows 86–91% of ^{32}P in site 1, 9–12% of ^{32}P in site 2 and 0–2% of ^{32}P in site 3 [1,3,6]. PDHP complex ($\alpha P \cdot \alpha\beta_2$) prepared with non-radioactive ATP, incorporates ^{32}P from [γ-^{32}P]ATP into sites 2 and 3 [3]. PDH(SP) complex [α(SP)$\alpha\beta_2$] incorporated ^{32}P into sites 2 and 3 in strictly analogous fashion. This was shown by the presence of ^{32}P in two phosphopeptides whose electrophoretic mobilities corresponded to the diphosphotetradecapeptide (sites 1 and 2) and to the phosphononapeptide (site 3) [1,3]. This indicates thiophosphorylation of site 1. When PDH^{32}P complex ($\alpha P \cdot \alpha\beta_2$) was incubated with ATPγS, ^{32}P moved from the phosphotetradecapeptide to the diphosphotetradecapeptide on high-voltage electrophoresis. This indicates thiophosphorylation of site 2 which has also been shown in [15]. The phosphorylation of site 3 was assumed from the following. PDH^{32}P complex ($\alpha P \cdot \alpha\beta_2$) was prepared with low specific activity [γ-^{32}P]ATP (4 dpm/pmol) and contained 0.51 nmol P/unit complex inactivated. It was then incubated for 90 min with 0.5 mM ATPγS (see section 2.1). The presumed PDHP$(SP)_2$ complex (recovered by centrifugation) was then incubated with high specific activity [γ-^{32}P]ATP (180 dpm/pmol) in an attempt to identify vacant phosphorylation sites. The incorporation (0.09 nmol P/unit PDH complex) was ⩽10% of the incorporation of ^{32}P into sites 2 and 3 of PDHP ($\alpha P \cdot \alpha\beta_2$). It is assumed therefore that sites 2 and 3 were thiophosphorylated.

3.2. *Effect of PDHP phosphatase on thiophosphoryl complexes*

Figure 2 shows that there was very little reactivation of PDH(SP) complex [α(SP)$\alpha\beta_2$] by ox-heart PDHP phosphatase. Reactivation over 30 min at high phosphatase only achieved 7% of the reactivation of PDHP complex ($\alpha P \cdot \alpha\beta_2$) achieved in 5 min under identical conditions (shown in fig.2). In further experiments it was shown that PDH(SP) complex [α(SP)$\alpha\beta_2$] is a competitive inhibitor of the release of $^{32}P_i$ from PDH^{32}P complex ($\alpha P \cdot \alpha\beta_2$) with ox-heart phosphatase. The K_m for PDHP was 15.9 ± 0.88 units/ml (PDH complex equiv.) and the K_i for PDH(SP) was 32.7 ± 1.53 units/ml (mean ± SEM; 5 conc. PDHP over 6.4–34.9 units/ml; PDH(SP) 34.9 units/ml; 8 obs. each conc.; measurements at 2.5 min, maximum hydrolysis 37%).

Figure 3 shows that ox-heart PDHP phosphatase released as $^{32}P_i$, 86% of the ^{32}P in PDH^{32}P $(SP)_2$ complex. This release of $^{32}P_i$ was associated with conversion of 12% of the inactive PDHP$(SP)_2$ complex to active complex. Release of $^{32}P_i$ was 4.36 ± 0.27 nmol P/unit active complex formed (r, 0.985). This may be compared with the release of 0.41 nmol P/unit active

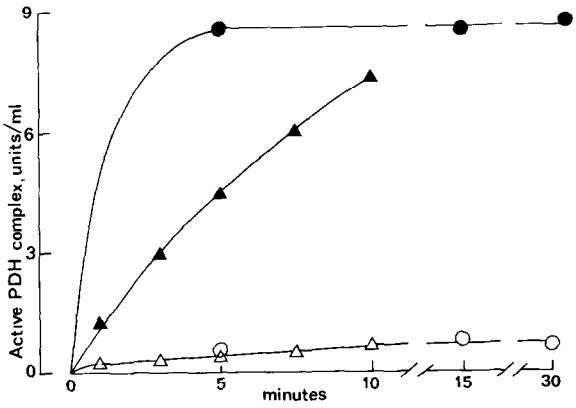

Fig.2. Incubations were made at 30°C in medium A (see section 2.2) (16 mM Mg^{2+}, 13 μM Ca^{2+}) with either PDHP complex (●,▲) or PDH(SP) complex (○,△) (9 units/ml). After 5 min preincubation, reaction initiated with 5 μl (▲,△) or 20 μl (●,○) of ox heart phosphatase. Samples taken for assay of PDH complex at times shown. Each point is mean of 3 obs. corrected for zero time PDH complex activity [3.5% in PDHP; 3.1% in PDH(SP)]. At all times $P < 0.001$ for differences [(▲)–(△)] and [(●)–(○)].

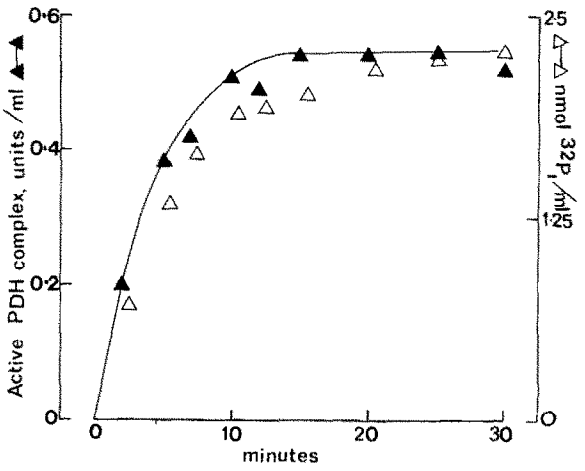

Fig.3. Incubations made at 30°C in medium A (see section 2.2) (16 mM Mg^{2+}, 13 μM Ca^{2+}) and 4.95 units $PDH^{32}P(SP)_2$ complex. After 5 min preincubation reaction initiated with 20 μl ox-heart PDHP phosphatase. Samples taken for assay of PDH complex (▲) or $^{32}P_i$ (△), at times shown. Total ^{32}P in the incubate was estimated at 30.5 min. The values for $^{32}P_i$ were corrected for $^{32}P_i$ in the $PDH^{32}P(SP)_2$ complex (0.21% of total). The complex was devoid of PDH complex activity. At the limit 12% of complex was reactivated and 86% of ^{32}P released as $^{32}P_i$. Points are means of 3 obs.

complex formed from PDHP complex ($\alpha P \cdot \alpha\beta_2$) [5]. The experiment was repeated (not shown in fig.3); 17% of complex was reactivated and release of $^{32}P_i$ was 3.91 nmol P/unit. Three interpretations are possible and these cannot readily be distinguished with techniques at present available.

(i) $PDH(SP)_2$ complex (i.e., PDH complex thiophosphorylated in sites 2 and 3) is active but has 12–17% of the activity of PDH complex.

(ii) PDHP complex ($\alpha P \cdot \alpha\beta_2$) contains 9–12% of phosphate in site 2 and $PDHP(SP)_2$ complex may therefore contain up to 12% of phosphate in site 2. If site 3 was incompletely thiophosphorylated, some reactivation could occur by removal of phosphate.

(iii) It is possible that PDHP phosphatase may remove thiophosphate from sites 2 and 3.

Little or no reactivation of bovine kidney $PDHP(SP)_2$ complex on removal of phosphate with bovine kidney phosphatase was observed [15]. The reason for the discrepancy between our findings and those in [15] is not apparent. The technique of selective phosphorylation of site 1 in [15] was less selective than the technique of discontinuous phosphorylation used here (25% of the ^{32}P incorporated in $PDHP(SP)_2$ in [15] was in sites 2 and 3). It was concluded in [15] that sites 2 and 3 may be inactivating sites. Evidence given in [4] has shown that site 3 plays no part in inactivation in vivo and that sites 2 and 3 inhibit reactivation by PDHP phosphatase [5,6].

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