ROLE OF MULTISITE PHOSPHORYLATION IN THE REGULATION OF OX KIDNEY PYRUVATE DEHYDROGENASE COMPLEX

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1. Introduction

The pyruvate decarboxylase $\alpha_2\beta_2$ tetramers (EC 1.2.4.1) of mammalian PDH (EC 1.2.4.1, EC 2.3.1.12, EC 1.6.4.3) are phosphorylated on three specific serine residues [1,2]. Inactivation involves incorporation of 1 P/$\alpha_2\beta_2$ (site 1) and complete phosphorylation involves incorporation of 3 P/$\alpha_2\beta_2$ (sites 2,3) [1,3]. Phosphorylation sites are only on the α-subunits [3,4]. Phosphorylation of sites 2,3 inhibits the reactivation of pig heart PDHP by its phosphatase [5,6]. This has not been confirmed in ox kidney PDHP [7]. Here we show that there is in fact a similar function for phosphorylation of sites 2,3 in both the ox kidney and pig heart PDHP preparations.

2. Experimental

2.1. *Materials*

Leupeptin was obtained from the Protein Res. Found., Osaka. Sources of other chemicals, biochemicals, radiochemicals and details of PDHP phosphatase preparation (ox heart) are given in [2,3,5,6,8,9]. PDH (ox kidney) was prepared essentially as in [9,10]. Kidneys were homogenized in 0.25 M sucrose/5 mM potassium phosphate/1 mM EDTA (pH 7.0) [10].

Abbreviations: PDH, pyruvate dehydrogenase complex; PDHP, pyruvate dehydrogenase phosphate complex of unspecified phosphorylation state; $PDHP_1$, PDH titrated to inactivation containing ~1 mol P/mol pyruvate decarboxylase $\alpha_2\beta_2$ tetramer; $PDHP_3$, fully phosphorylated PDH containing 3 mol P/mol $\alpha_2\beta_2$; MOPS, 2-(*N*-morpholino)propane sulphonic acid; DTT, dithiothreitol; EGTA, ethanedioxybis (ethylamine)-tetraacetic acid; K_d, dissociation constant; *n* no. obs.

Mitochondria were collected [10] and washed with 0.1 mM DTT and then 20 mM potassium phosphate/0.2 mM DTT (pH 7.0). They were resuspended in the latter buffer (40 ml/kidney), frozen and thawed, and treated as in [9] with the following exceptions: (a) pH 6.1 steps were replaced by pH 6.2 steps; (b) PDH was not completely precipitated at pH 5.4 and therefore 0.02 vol. 50% (w/v) polyethylene glycol 6000 was added; (c) 2-mercaptoethanol was replaced by DTT. The final yield of PDH was 10–20 units/kidney and it was stored at –20°C where its activity was stable for ⩾1 month. PDH was ⩾95% homogeneous by SDS–polyacrylamide disc gel electrophoresis [3]. Molecular weights of subunits were: lipoyl acetyltransferase, 77 200 ± 700; lipoyl dehydrogenase, 55 400 ± 400; pyruvate decarboxylase α subunit, 37 500 ± 400; β subunit, 31 800 ± 300 (mean ± SEM for n = 18). The values for the α and β subunits are lower than obtained in [3,4]. Contrary to [11], inclusion of leupeptin (2 μg/ml) in stages from mitochondrial resuspension prior to freezing and thawing onwards did not affect the gel pattern.

2.2. *Methods*

PDH was assayed as in [9]. (One unit forms 1 μmol NADH/min at 30°C.)

PDH was phosphorylated to completion with [γ-^{32}P]ATP as in [3,5]. Inactivation took 1–2 min and complete phosphorylation 20 min. Incorporation was 1.43 ± 0.03 nmol P/unit enzyme activity inactivated (mean ± SEM, n = 4). This preparation will be called $PDHP_3$.

PDH was phosphorylated to 95–98% inactivation as in [3,5] by repetitive addition of limiting quantities of [γ-^{32}P]ATP. Incorporation was 0.57 ± 0.04 nmol P/unit enzyme activity inactivated (mean ± SEM,

$n = 4$). This preparation will be called $PDHP_1$.

After phosphorylation, 0.05 vol. 0.2 M EDTA (pH 7.5) were added to the PDHP preparations which were collected by centrifugation at 150 000 × *g* for 2 h. PDHP was redissolved in ~1 ml 20 mM potassium phosphate/2 mM DTT (pH 7.0) (30–40 units equiv./ml) and dialysed overnight against the same buffer or against 50 mM MOPS/2 mM DTT (pH 7.0). PDHP preparations were standardized by complete reactivation and dephosphorylation with PDHP phosphatase [5,6].

Dephosphorylations were carried out with PDHP and PDHP phosphatase dialysed into potassium phosphate or MOPS buffer (see above). For dephosphorylation in potassium phosphate buffers, the final concentrations were: 25 mM free Mg^{2+} (added as $MgCl_2$):10 mM total potassium phosphate:25 mM Tris–HCl:PDHP equiv. 2–4 units/ml and PDHP phosphatase. For dephosphorylation in MOPS buffers, final concentrations were 10 mM free Mg^{2+} (added as $MgCl_2$):50 mM MOPS:PDHP and PDHP phosphatase. The rate of reactivation of PDHP to PDH was linear for a minimum of 2 min. Free Ca^{2+} concentrations were buffered using 20 mM total EGTA and various computed $CaCl_2$ concentrations [5,8]. For 0.1 mM and 0.5 mM free Ca^{2+}, EGTA was omitted. The final pH was 7.0. Incubations were done in 50–100 μl at 30°C and were initiated by addition of PDHP after 1 min preincubation. After various times (1–4 min) samples (10–20 μl) were withdrawn and assayed for PDH activity [9] or trichloroacetic acid-soluble ^{32}P [12]. Zero-time blanks were subtracted to allow for PDH activity in the PDHP (<10% of total for PDHP, and <1% for $PDHP_3$) and trichloroacetic acid-soluble ^{32}P.

3. Results

3.1. *Phosphate distribution in PDHP preparations*

The distribution of phosphate between the 3 phosphorylatable serine residues was examined [13,14] assuming monophosphorylated tetradecapeptide was phosphorylated only in site 1. For $PDHP_1$, it was site 1 (inactivating site), 71%; sites 1 + 2, 25%; site 3, 3%. For $PDHP_3$, it was site 1, 7%; sites 1 + 2, 75%; site 3, 17%. The distribution was not as discrete as for pig heart PDHP [6,14]. The theoretical distribution for $PDHP_3$ is sites 1 + 2, 67%; site 3, 33%. It was considered possible that sites were not dephosphorylated fully during the PDH preparation. However, incubation of purified PDH with PDHP phosphatase followed by three 2 h centrifugations at 150 000 × *g* to remove phosphatase and subsequent phosphorylation did not alter phosphate distribution.

3.2. *Effects of the buffer system on the rate of reactivation of $PDHP_1$ and $PDHP_3$*

Results are shown in table 1. They confirm that the ox kidney PDHP behaves similarly to the pig

Table 1
Effects of buffer system on the rates of reactivation of $PDHP_1$ and $PDHP_3$

	Incubation (min)	Formation of active PDH (units/ml) Mean ± SEM ($n = 3$)		Ratio $PDHP_1/PDHP_3$
		$PDHP_1$	$PDHP_3$	
20 mM K-phosphate/	2	0.62 ± 0.07	0.20 ± 0.01[a]	3.10 ± 0.33
0.38 μM Ca^{2+}	4	0.85 ± 0.06	0.24 ± 0.01[c]	3.55 ± 0.27
20 mM K-phosphate/	1	1.14 ± 0.15	0.49 ± 0.04[a]	2.31 ± 0.31
100 μM Ca^{2+}	2	2.12 ± 0.02	1.28 ± 0.07[c]	1.66 ± 0.08
50 mM MOPS/	2	0.54 ± 0.03	0.103 ± 0.004[b]	5.19 ± 0.14
0.38 μM Ca^{2+}	4	1.34 ± 0.05	0.32 ± 0.01[c]	4.15 ± 0.15
50 mM MOPS/	1	0.30 ± 0.05	0.22 ± 0.01	1.38 ± 0.20
100 μM Ca^{2+}	2	0.46 ± 0.06	0.36 ± 0.02	1.24 ± 0.15

Statistical significance for $PDHP_3$ incubations versus $PDHP_1$ incubations is: [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$ by Student's *t*-test.

Incubation media were as described in section 2.2. Free Mg^{2+} was 25 mM for K-phosphate incubations and 10 mM for MOPS incubations. For K-phosphate incubations PDHP was 4 units/ml; for MOPS incubations PDHP was 3 units/ml

heart complex [5,6]. In potassium phosphate buffers, the rates of reactivation of $PDHP_1$ and $PDHP_3$ were significantly different both at 0.38 μM and 100 μM free Ca^{2+}. The ratios of the rates of reactivation are 1.66–3.55. In MOPS buffer, there were significant differences in the rates of reactivation only at 0.38 μM free Ca^{2+}. At 100 μM free Ca^{2+}, no significant differences were observed. The latter conditions (MOPS, 10 mM Mg^{2+}, 100 μM Ca^{2+}) were used by workers unable to detect differences in reactivation rate of $PDHP_1$ versus $PDHP_3$ [7]. There was no difference in the rates of reactivation under these conditions even though the % reactivation/min was relatively low compared to other experiments. (In the MOPS/100 μM Ca^{2+} experiment, the amount of PDHP phosphatase was reduced to decrease the possibility that differences in reactivation rate might be obscured by too rapid a rate of reactivation.) The ratios of the rates of reactivation for $PDHP_1/PDHP_3$ were lower for ox kidney than described [6] for pig heart PDH. This may reflect a less advantageous distribution of phosphate in sites 2,3 in ox kidney PDHP (16% for $PDHP_1$ against 55% in $PDHP_3$) as compared to pig heart PDHP (9% for $PDHP_1$ against 66% for $PDHP_3$) [14].

3.3. *Effects of free Ca^{2+} concentration on the rate of reactivation and dephosphorylation of PDHP by PDHP phosphatase*

Results are shown in fig.1. For reactivation of PDHP, release of activity was significantly (at least $p < 0.05$) quicker with $PDHP_1$ than with $PDHP_3$ at all Ca^{2+} concentrations. For dephosphorylation of PDHP, P release was always about twice as rapid from $PDHP_3$ as from $PDHP_1$. These results agree with observations using pig heart PDHP [5,6]. Apparent K_d for free Ca^{2+} and maximal velocities were computed [15]. For reactivation, app. K_d values were $PDHP_1$, 2.05 ± 0.27 μM; $PDHP_3$, 4.67 ± 0.99 μM. V_{max} were $PDHP_1$, 1.18 ± 0.03 units . ml^{-1} . min^{-1}; $PDHP_3$, 0.67 ± 0.04 units . ml^{-1} . min^{-1}. For dephosphorylation, app. K_d values for free Ca^{2+} were: $PDHP_1$, 3.49 ± 0.68 μM; $PDHP_3$, 2.60 ± 0.41 μM. V_{max} were $PDHP_1$, 0.54 ± 0.03 nmol P . min^{-1} . ml^{-1}; $PDHP_3$, 1.02 ± 0.04 nmol P . min^{-1} . ml^{-1}. Apparent K_d values computed above were not significantly different. However, at Ca^{2+} concentrations below app. K_d (0.38 and 1.70 μM), the ratios of velocity: V_{max} for PDHP activation were significantly different for $PDHP_1$ and $PDHP_3$. This implies a difference in app.

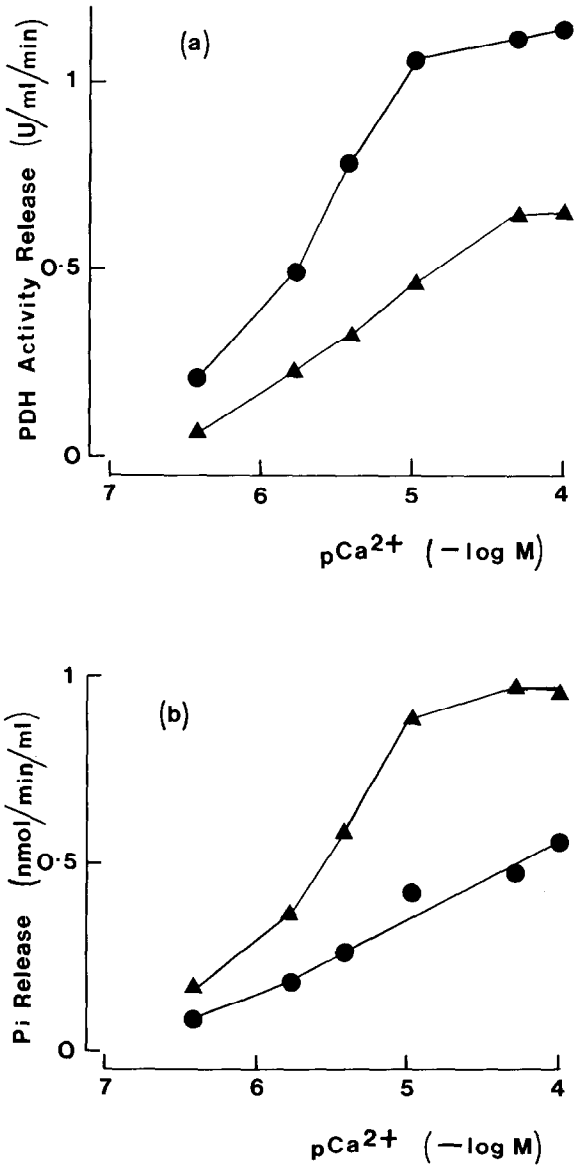

Fig.1. Reactivation and dephosphorylation of ox kidney PDHP by PDHP phosphatase at various Ca^{2+} concentrations. Incubations were carried out as described in section 2.2 in K-phosphate buffers at 30°C. The incubations were total vol. 50 μl. PDHP phosphatase (5 μl) was added. After 1 min preincubation, the reaction was initiated by addition of 10 μl $PDHP_1$ (●) or $PDHP_3$ (▲) to final PDHP conc equiv. 4 units/ml. Samples were removed for assay of PDH activity and trichloroacetic acid-soluble ^{32}P. In (a) the rate of release of PDH activity is plotted against pCa^{2+} (−log [computed free Ca^{2+}]). In (b) the rate of release of trichloroacetic acid-soluble ^{32}P from PDHP is plotted against pCa^{2+}. Results were corrected for PDH activity and trichloroacetic acid-soluble ^{32}P present in PDHP preparations.

K_d for Ca^{2+} for reactivation for $PDHP_1$ and $PDHP_3$. Phosphorylation of sites 2,3 increases the app. K_d for Ca^{2+} for reactivation (see also [6]). The ratio of maximal velocities of reactivation for $PDHP_1$: $PDHP_3$ was 1.76; for dephosphorylation it was 0.53. The latter presumably reflects the lower concentration of phosphate groups in the $PDHP_1$ experiments and/or the nature of the dephosphorylation mechanism. The app. K_d values for Ca^{2+} were lower than values recently obtained for pig heart PDHP [6]. This suggests that the differential rates of reactivation of $PDHP_1$ and $PDHP_3$ by PDHP phosphatase are independent of app. K_d. The differences between K_d values may have arisen because of differences in methods of PDH preparation [6].

4. Discussion

These results demonstrate that the phosphorylation of sites 2,3 in ox kidney PDH (see table 1) inhibit the rate of reactivation of PDHP by PDHP phosphatase. This behaviour was observed initially with pig heart PDHP [5] and these observations have been greatly amplified in [6]. Other workers have failed to observe inhibition of the rate of reactivation with ox kidney PDH [7]. Our results support the conclusion [6] that the failure to observe inhibition [7] is caused by the use of a different buffer system (MOPS/100 μM free Ca^{2+}) from that used in [5]. Using potassium phosphate or MOPS/0.38 μM Ca^{2+}, reactivation of $PDHP_1$ was always more rapid than $PDHP_3$ (table 1, fig.1.). The difference between results [5,7] is not caused by a species or tissue difference in the behaviour of pig heart and ox kidney PDH.

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