# The roles of intrinsic kinase and of kinase/activator protein in the enhanced phosphorylation of pyruvate dehydrogenase complex in starvation

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Extracts of heart mitochondria from fed and from 48 h starved rats subjected to gel filtration on Sephacryl S-300 gave 4 major protein peaks. Pyruvate dehydrogenase complex eluted in the void volume and was assayed for intrinsic pyruvate dehydrogenase kinase activity which was increased approximately 3-fold by 48 h starvation of the rat. A second fraction, containing peaks 2 and 3 which overlapped, enhanced the activity of the intrinsic kinase and corresponds to kinase/activator protein described previously. Its activity was increased 1.5-fold by starvation.

*Pyruvate dehydrogenase complex* *Phosphorylation* *Kinase/activator* *Pyruvate dehydrogenase kinase* *Starvation* *Inactivation by phosphorylation*

## 1. INTRODUCTION

In animals the pyruvate dehydrogenase (PDH) complex is inactivated by phosphorylation of the $\alpha$-subunit of its decarboxylase component by PDH kinase intrinsic to the complex; PDH phosphatase effects dephosphorylation and reactivation [1]. In 48 h starved or diabetic rats phosphorylation and inactivation of the complex inhibits pyruvate and glucose oxidation [2,3]. This is apparently effected by increased activity of PDH kinase [4–6]. The effect of starvation and of diabetes requires oxidation of lipid fuels being reversed by inhibitors of fatty acid oxidation [7]. Effects of lipid fuels may be mediated by an increased mitochondrial ratio of [acetyl CoA]/[CoA] which activates PDH kinase [1,4,8–10]. A further and perhaps more fundamental mechanism of PDH kinase activation is independent of the acetyl CoA/CoA ratio and persists into mitochondria and mitochondrial extracts [4–6]. Evidence has been given that this may involve a protein, apparently induced by cytoplasmic protein synthesis and which remains in the supernatant when PDH complex is removed from mitochondrial extracts by sedimentation (150000 $\times$ *g*) [11]. This has been termed kinase/activator because it could either be additional free PDH kinase and/or activator of PDH kinase [11]. It was not possible in these studies to ascertain whether the activity of PDH kinase, intrinsic to the PDH complex, is enhanced by starvation in the absence of kinase/activator. This has now been achieved as a result of separation by gel filtration; PDH complex from starved rats possesses enhanced PDH kinase activity.

## 2. EXPERIMENTAL

Source of rats and details of feeding are as in [11]. Rat heart mitochondria were isolated (nagarse or Polytron methods) and incubated in KCl medium for 15 min at 30°C to convert inactive complex into active complex [4,5]. Extracts (40 mg protein/ml) were prepared by freezing and thawing [5] in buffer A [50 mM potassium phosphate/10 mM EGTA/1 mM phenylmethane sulphonyl fluoride/0.3 mM 7-amino-1-chloro-3-L-tosylamidoheptan-2-one/1 mM benzamidine/2

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mM dithiothreitol/1% (v/v) ethanol (pH 7)]. Sephacryl S-300 was from Pharmacia. Sources of other materials are in [3,12]. PDH complex was assayed spectrophotometrically by coupling to arylamine acetyltransferase [9]. PDH kinase was assayed routinely by the ATP dependent loss of PDH complex activity at 30°C. Assays were in buffer A (plus oligomycin B 25 $\mu g \cdot ml^{-1}$ and 1 mM $MgCl_2$) with ATP to 0.5 mM. PDH kinase was assayed additionally by incorporation of $^{32}P$ into PDH complex from [$\gamma$-$^{32}P$]ATP [9,11]. PDH phosphatase does not interfere in these assays [5,11,12]. Sephacryl S-300 chromatography was in buffer A (elution, 14 ml/h). Kinase/activator was located after gel filtration on Sephacryl by its activating effect on PDH kinase activity in pig heart PDH complex at 0.18 units/ml [11].

## 3. RESULTS AND DISCUSSION

### 3.1. *Gel filtration of rat heart mitochondrial extracts on Sephacryl S-300; ATP dependent inactivation of PDH complex*

When rat heart mitochondrial extracts were chromatographed on Sephacryl S-300 PDH complex eluted at the void volume and kinase/activator in a later fraction. The profile shown in fig.1 is typical of 10 such profiles. Four major peaks at $A_{280nm}$ were observed with elution volumes of 37–43 ml (peak 1, fraction 1 in fig.1), 51–57 ml (peak 2, shoulder to fraction 2 in fig.1), 58–65 ml (peak 3, main peak in fraction 2 in fig.1), 85–99 ml (peak 4) and 100–108 (peak 5) (peaks 4 and 5 not shown in fig.1). Assay showed that fraction 1 in fig.1 contained the PDH complex. The tubes with the highest activity (usually 3 tubes) were pooled for PDH kinase assays. By assay with pig heart complex, kinase/activator was shown to be present in tubes 54–63 of fraction 2 (fig.1); the remaining fractions were devoid of kinase/activator. The whole of fraction 2 in fig.1 was used for studies with kinase/activator. There was no difference in protein content of fraction 2 between fed and starved (not shown).

The results of PDH kinase assays are shown in table 1 as the pseudo first order rate constants for ATP-dependent inactivation of PDH complex. PDH kinase activity is dependent on the concentration of PDH complex in the assay (compare lines 3 and 4, table 1; other results not shown; see also [11]). Care was taken to compare fed and starved at comparable concentrations of PDH complex (see columns 2 and 3, table 1). In fresh mitochondrial extracts PDH kinase activity in the 48 h starved was 3.7-fold greater than that of the fed (line 1, table 1). After storage overnight in liquid $N_2$ and clarification by centrifugation, PDH kinase activity increased (significant only in the fed); the effect of starvation was not changed significantly (3.1-fold, line 2, table 1). After gel filtration on Sephacryl S-300 PDH kinase activity in fed and starved was decreased by 75% but the effect of starvation was not changed significantly (2.8-fold, line 3, table 1). Thus, the enhanced PDH kinase activity in extracts of mitochondria induced by 48 h starvation is exhibited by the fraction containing PDH complex after gel filtration on

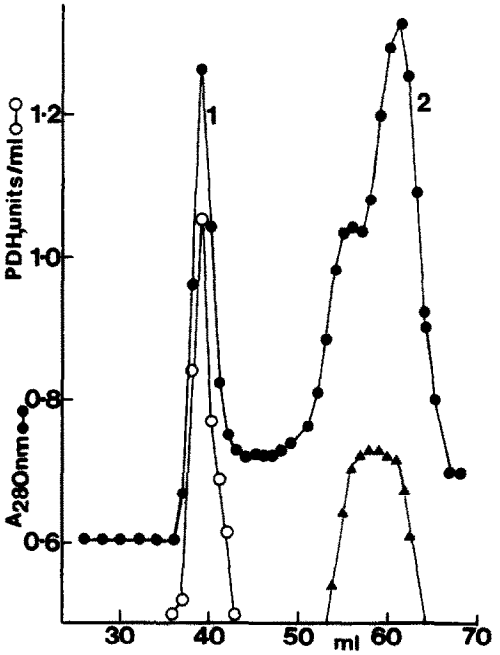


Fig.1. Gel filtration (1 ml fractions) of rat heart mitochondrial extract (84 mg protein; 5.8 units of PDH complex; 1.16 ml) on Sephacryl S-300 (bed volume 130 ml) in Buffer A (see section 2). Fraction 1 (37–43 ml) contained PDH complex (○); Fraction 2 (53–65 ml) contained kinase/activator (▲, arbitrary units not shown). On the same column, but in separate runs, $M_r$ markers (0.5 ml) gave peaks at 41 ml ($M_r$ 670000), 51 ml ($M_r$ 158000) and 61 ml ($M_r$ 44000). Kinase activator eluted at 54–63 ml (apparent $M_r$ at the mid-peak 73000).

Table 1

Effect of starvation (48 h) and of gel filtration (Sephacryl S-300) on the PDH kinase activity of PDH complex extracted from rat heart mitochondria

| Fraction | PDH complex (munits/ml) | | PDH kinase activity (pseudo first order rate constant $min^{-1}$; mean ± SE, 40 obs) | | Starved/fed (mean ± SE of ratio) |
|---|---|---|---|---|---|
| | Fed | Starved | Fed | Starved | |
| Fresh extract | 275 | 265 | 0.43 ± 0.04 | 1.61 ± 0.10[a] | 3.73 ± 0.29[a] |
| Extract loaded | 190 | 210 | 0.65 ± 0.08[c] | 1.74 ± 0.10 | 3.06 ± 0.37[a] |
| Sephacryl eluate | | | | | |
| Fraction 1 | 204 | 192 | 0.15 ± 0.03[c] | 0.38 ± 0.04[a,c] | 2.75 ± 0.51[a] |
| Fraction 1 | 412 | 412 | 0.36 ± 0.03 | 1.35 ± 0.03[a] | 3.76 ± 0.26[a] |
| Fractions 1 + 2 | 205 | 205 | 1.12 ± 0.12[b,c] | 2.73 ± 0.63[a,b] | 2.44 ± 0.45[a] |

[a] $P < 0.01$ for effect of starvation
[b] $P < 0.001$ for effect of Fraction 2
[c] $P < 0.01$ for difference from fresh extract

PDH kinase activity was assayed by the rate of inactivation of PDH complex by 0.5 mM ATP/1 mM $MgCl_2$ in buffer A containing 25 μg/ml oligomycin B (samples at 4 time points up to 2 min). Results are means of 2–4 determinations in each of 3 experiments employing extracts of mitochondria isolated from 12 rat hearts (fed or 48 h starved). Extract loaded (onto Sephacryl) had been stored overnight in liquid $N_2$ and clarified (after thawing) at 33000 × g, 15 min). The concentration of fraction 2 was 117 μl in total volume of 222 μl; for definition of fractions 1 and 2 see fig.1

Sephacryl S-300 but the overall PDH kinase activity was decreased by gel filtration in both fed and starved.

Sephacryl S-300 chromatography would be expected to separate kinase/activator protein from PDH complex ($M_r$, PDH complex approx. $10^7$; kinase/activator approx. $10^5$). When fractions 2–5 (see above) were tested for their ability to enhance PDH kinase, only fraction 2 (fig.1) possessed activity (other results not shown). As shown in table 1, line 5, fraction 2 (117 μl out of total assay volume of 222 μl) increased PDH kinase activity in fraction 1 to a value greater than that seen in mitochondrial extracts. It is suggested that this overshoot results from the removal of low-$M_r$ inhibitors of PDH kinase by gel filtration (e.g., ADP, thiamin pyrophosphate). When corresponding fractions 1 and 2 were combined (i.e., fed/fed and starved/starved) the effect of starvation (2.4-fold) was not changed significantly by inclusion of kinase/activator (line 5, table 1). The effect of fraction 2 from starved rats on PDH complex from fed or starved rats was, however, significantly greater than that of fraction 2 from fed rats. The results ($min^{-1}$, mean ± SE for 3–4 obs) were: (fed complex + fed fraction 2) 1.10 ± 0.02, (fed complex + starved fraction 2) 1.40 ± 0.09 ($P < 0.05$ for difference); (starved complex + fed fraction 2) 1.93 ± 0.09, (starved complex + starved fraction 2) 2.73 ± 0.11 ($P < 0.01$ for difference). Therefore, when tested on the same PDH complex, fraction 2 from starved rats possessed greater activity than fraction 2 from fed rats. These results are comparable to those of an earlier study [12] in which mitochondrial extracts were used as the source of PDH complex and a high speed supernatant fraction as the source of kinase/activator.

### 3.2. *Gel filtration of rat heart mitochondrial extracts on Sephacryl S-300; formation of [$^{32}$P]phosphorylated PDH complex*

It was important to establish that the effects of starvation and of kinase/activator on ATP-dependent inactivation of PDH complex were associated with phosphorylation, by measuring incorporation of $^{32}P$ from [$\gamma$-$^{32}P$]ATP into the complex. This has already been shown for mitochondrial extracts [5]. Fig.2 shows incorporation of $^{32}P$ from [$\gamma$-$^{32}P$]ATP into PDH complexes isolated by gel filtration from rat heart mitochondrial ex-

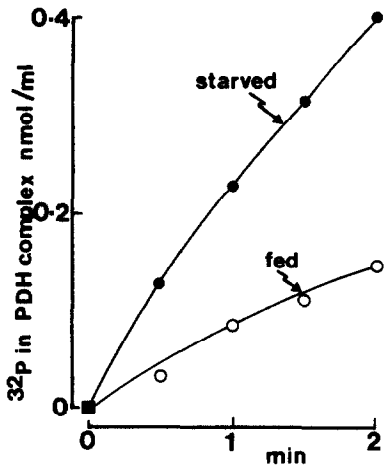


Fig.2. Incorporation of $^{32}$P from 0.5 mM [$\gamma$-$^{32}$P]ATP into PDH complex (0.3 units/ml) isolated from heart mitochondrial extracts of fed (○) or 48 h starved (●) rats by gel filtration on Sephacryl S-300 (see fig.1). Other conditions of incubation as in table 1. Each point is the mean of 6 obs. For each time point, $P < 0.001$ for the effect of starvation. Mean ratio starved/fed was 3.02 ± 0.19 ($P < 0.001$ for difference from unity).

tracts. Incorporation was enhanced by 48 h starvation (ratio starved/fed 3.02 ± 0.19; $P < 0.001$ for difference from unity). The relationships between phosphate incorporation and inactivation were as predicted from data for rat heart complex in [3] (not shown).

The effects of fraction 2 (fig.1) on $^{32}$P incorporation into PDH complexes separated by gel filtration (0.2 units/ml) at a single time point (35 s) using conditions of incubation given in table 1 (ATP 286 dpm/pmol) were as follows (mean ± SE for 6 obs; pmol $^{32}$P incorporated/ml): PDH complex of fed rats: alone 42 ± 4; with fraction 2 of fed rats, 62 ± 3; with fraction 2 of starved rats, 101 ± 4.

PDH complex of starved rats: alone, 57 ± 2; with fraction 2 of fed rats, 96 ± 3; with fraction 2 of starved rats, 146 ± 3.

All increases induced by starvation or by fraction 2 were significant ($P < 0.01$). The ratio (starved/fed) for PDH complex alone was 1.4 ± 0.11; this was smaller than the ratio in fig.2 (approx. 3). This is assumed to be an effect of more prolonged storage of PDH complex (fraction 1) at −80°C prior to assay with fraction 2. The ratio (starved/fed) for mixtures of corresponding fractions 1 + 2 was 2.4 ± 0.13. This ratio is significantly greater than the ratio in the absence of fraction 2 (1.4 ± 0.11; $P < 0.001$).

## 4. CONCLUSIONS

This study shows that PDH complex in rat heart mitochondrial extracts can be separated from kinase/activator by gel filtration on Sephacryl S-300. The effect of kinase/activator on PDH kinase intrinsic to PDH complex prepared by gel filtration was enhanced by starvation. In this respect the results of separating kinase/activator from PDH complex by gel filtration (this study) and differential centrifugation [11,12] are similar. This study shows for the first time that the activity of PDH kinase intrinsic to the PDH complex is enhanced by 48 h starvation of the rat. The importance of this observation lies in the stable nature of the change induced by starvation. This persisted through isolation and incubation of mitochondria, preparation of extracts and gel filtration – some 24 h of manipulation. This indicates that the mechanism involves either an increase in the number of PDH kinase molecules/molecule of holocomplex or a stable form of activation of which covalent modification is the most obvious example. If the change induced by starvation is due to covalent modification then the mitochondria lack enzyme(s) capable of reversing it under the conditions used here.

The PDH kinase activity of PDH complex was decreased following separation from kinase/activator and was restored by recombination. Whether the same mechanism underlies both of the effects of starvation, i.e., on the activity of PDH kinase intrinsic to the PDH complex and of kinase/activator, remains to be established.

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