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Evidence that rat liver pyruvate dehydrogenase kinase activator protein is a pyruvate dehydrogenase kinase

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It is shown here that rat liver pyruvate dehydrogenase (PDH) kinase activator protein (KAP) catalyses ATP-dependent inactivation and [^{32}P]phosphorylation of pig heart PDHE1 and of yeast (*Saccharomyces cerevisiae*) PDH complex devoid of PDH kinase activity, that fluorosulphonylbenzoyladenosine inactivates rat liver KAP and the intrinsic PDH kinase of rat liver PDH complex, and that KAP, like PDH kinase, is inactivated by thiol-reactive reagents. It is concluded that KAP is a free PDH kinase.

INTRODUCTION

Mammalian mitochondrial pyruvate dehydrogenase (PDH) complexes are inactivated by phosphorylation of the E1α component with MgATP, catalysed by a PDH kinase intrinsic to the complex [1–3]. Rat heart, liver and skeletal-muscle mitochondria contain a protein activator of PDH kinase (KAP) which can be separated from PDH complex by gel filtration or ultracentrifugation [4–6]. Starvation or alloxan diabetes increase the specific activity of KAP in rat heart and liver approx. 3-fold in 24–48 h, and experiments with hepatocytes in tissue culture have indicated that their effects may be mediated by fatty acids and by cyclic AMP [5,7–9].

Evidence given here shows that KAP is a PDH kinase. Pig heart pyruvate dehydrogenase (i.e. the E1 component of pig heart PDH complex), which as made contains PDH kinase [10,11], has been freed of PDH kinase by ion-exchange chromatography and treatment with *N*-ethylmaleimide (NEM). Yeast (*Saccharomyces cerevisiae*) PDH complex, which is devoid of PDH kinase [12,13], is phosphorylated and inactivated by mammalian PDH kinases, because a mammalian phosphorylation-site sequence is present in its E1 component. We show here that KAP phosphorylates and inactivates pig heart PDHE1 and yeast PDH complex. Furthermore, the PDH kinase activity of KAP was abolished by fluorosulphonylbenzoyladenosine (FSBA), which forms an adduct with the ATP-binding site of protein kinases, and this inactivation was inhibited in turn by p[NH]ppA, which is a known competitive inhibitor (versus ATP) of PDH kinase [14–17]. KAP, like PDH kinase intrinsic to the PDH complex [11], was inactivated by the thiol-reactive reagents NEM and *p*-chloromercuribenzoate (PCMB) [PCMB reversed by dithiothreitol (DTT)].

EXPERIMENTAL

Materials

Details relating to male Wistar rats (source, feeding), and sources of chemicals, biochemicals, [γ-^{32}P]ATP, Centriflo cones, Sephacryl and Sephadex are as in refs. [5,7,10–12]. In addition, NEM, PCMB, 8-azido-ATP and FSBA were from Sigma Chemical Co., Poole, Dorset, U.K., and freshly grown *S. cerevisiae* was given by British Fermentation Products, Felixstowe and Reading, U.K. PDH complex was purified from pig hearts [10] and gel-filtered on Sephacryl S300 to remove KAP [5].

Pyruvate dehydrogenase (PDHE1) and dihydrolipoate acetyltransferase (E2) were separated from pig heart PDH complex by the method of Pettit & Reed [18]. The resulting preparation of PDHE1 was largely freed of PDH kinase (> 98%) by two successive ion-exchange-chromatographic runs on DEAE-cellulose. The column (bed vol. 5 ml) was equilibrated with 10 mM-potassium phosphate, pH 7, and the PDHE1 was eluted with a linear gradient of 10–250 mM-potassium phosphate, pH 7. The last traces of PDH kinase were inactivated by incubation with 2 mM-NEM (2 min, 30 °C), followed by addition of DTT to 20 mM; 70% of PDH activity was retained after NEM treatment.

Yeast PDH complex was purified from extracts of *S. cerevisiae* by the method of Uhlinger *et al.* [13] up to the end of hydroxyapatite chromatography. The yeast extracts were prepared either with a French Press [12] or with glass beads in a Waring Blendor [19]. Removal of proteinases was further improved by an additional step (chromatography on haemoglobin–agarose [20]) immediately after protamine sulphate treatment). The resulting preparations had a specific activity of approx. 18 units/mg, and on SDS/PAGE displayed five bands, as shown in [12,13] (E2, E3, X, E1α and Eβ).

KAP was partially purified from extracts of liver mitochondria of fed or 48 h-starved rats [5] by three methods. Choice of method was dictated by scale of preparation (B, C > A) and yield (C > B). In method A, KAP was separated from PDH complex by gel filtration of mitochondrial extracts on Sephacryl S300 [5]. In method B, PDH complex and KAP were co-precipitated from mitochondrial extracts at pH 6.5 with 5% (w/v) poly(ethylene glycol) 6000; the precipitate was taken up in mitochondrial extraction buffer, and KAP was separated by centrifugation at 150000 *g* for 90 min, precipitated from the supernatant by addition of solid $(NH_4)_2SO_4$ (343 mg/ml) at 4 °C, taken up in 1–3 ml of extraction buffer, and gel-filtered on Sephadex G-150 (superfine grade; bed volume 120 ml). In method C, the poly(ethylene glycol) precipitate was dissolved in extraction buffer, clarified (40000 *g* for 20 min) and KAP was separated from PDH complex on Sephacryl S300. If necessary, KAP preparations were concentrated by $(NH_4)_2SO_4$ precipitation, followed by dialysis. Rat liver PDH complex was partially purified from the 150000 *g* precipitate obtained in method B as in [5].

Abbreviations used: PDH, pyruvate dehydrogenase; KAP, PDH kinase activator protein; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; FSBA, fluorosulphonylbenzoyladenosine; p[NH]ppA, adenosine 5′-[$\beta\gamma$-imido]triphosphate.

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Assays

PDH complexes were assayed spectrophotometrically by monitoring NAD^+ reduction at 340 nm or (when lactate dehydrogenase was present) by coupling to arylamine acetyltransferase as in [14]. PDHE1 was assayed as PDH complex after recombination with an excess of E2 and E3.

PDH kinase was assayed by the rate of ATP-dependent inactivation of PDH complexes or PDHE1 by using conditions given in [5], activity being computed as the apparent first-order rate constant [4,5]. Routinely, samples were taken for assay of PDH complex or PDHE1 activity at four times chosen to yield approx. 75% inactivation by the last time point; control incubations in the absence of ATP were included. PDH kinase activity was also assayed by the rate of incorporation of ^{32}P from [γ-^{32}P]ATP into PDH complexes or PDHE1 as in [13]. Activity of KAP was assayed by its effects on activity of PDH kinase [5] by including ^{32}P assay blanks with KAP or PDH complexes or PDHE1 alone. In screening column fractions for KAP, a semi-quantitative assay was employed, with a single fixed time of incubation with ATP of 1 min. KAP preparations were standardized by PDH kinase assays employing ATP-dependent inactivation of pig heart PDH complex; a unit of KAP is defined as the amount of KAP required to give $t_{0.5} = 30$ s, i.e. an apparent first-order rate constant of 1.4.

Protein was assayed with Coomassie Blue [21]. ATPase activities of PDH complex and of KAP were assayed as in [14], and were < 0.04% hydrolysis of ATP/min under the conditions of the PDH kinase assays.

RESULTS AND DISCUSSION

PDH kinase activity of KAP preparations, assayed with pig heart PDHE1 and with yeast PDH complex

Fig. 1 shows that KAP from livers of fed or 48 h-starved rats induces [^{32}P]phosphorylation of pig heart PDHE1 devoid of PDH kinase activity, and also that the rate is dependent on KAP concentration and that KAP from 48 h-starved rats exhibited greater activity. At higher KAP concentrations phosphorylation reached a maximum, and the limit incorporations were comparable with those observed with pig heart PDH kinase in [10]. This allowed approximate apparent first-order rate constants to be calculated. This limit phosphorylation corresponds to complete occupancy of site 1 and approx. 50% occupancies of sites (2+3) [10]. Comparable results were obtained by measuring ATP-dependent inactivation of PDHE1. The apparent first-order rate constants for [^{32}P]phosphorylation and ATP-dependent inactivation are given in the legend to Fig. 1.

KAP from livers of 48 h-starved rats possessed PDH kinase activity with respect to [^{32}P]phosphorylation and inactivation of *S. cerevisiae* PDH complex (Fig. 2). Rates were independent of the concentration of PDH complex over the range 0.5–10 units/ml. The apparent first-order rate constants for ATP-dependent inactivation of yeast complex with KAP (approx. 0.03 min^{-1}; see Fig. 2) were substantially less than for pig heart PDH complex (approx. 3.68 min^{-1}). Fig. 3 shows that the PDH kinase activity of rat liver KAP in respect of [^{32}P]phosphorylation of *S. cerevisiae* PDH complex was enhanced approx. 6-fold by starvation. Comparable observations have been made with respect to the effect of starvation in assays utilizing ATP-dependent inactivation of yeast PDH complex (results not shown). The effect of starvation to increase the activity of KAP is apparently greater in assays with yeast complex than in assays with pig heart PDH complex (which contains intrinsic PDH kinase (results not shown).

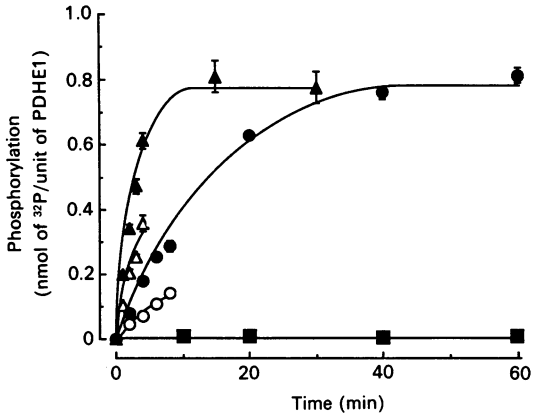

Fig. 1. [^{32}P]Phosphorylation of pig heart pyruvate dehydrogenase (PDHE1) by KAP from livers of fed (○, ●) and 48 h-starved (△, ▲) rats

KAP was prepared (method A) and pig heart PDHE1 was purified from pig heart PDH complex and freed of PDH kinase as described in the Experimental section. Concentrations were: [γ-^{32}P]ATP, 0.5 mM (155 d.p.m./pmol); PDHE1, 0.5 unit/ml (based on activity of PDH complex after reconstitution with E2 and E3); KAP (v/v), 0 (■), 12.5% (○, △), or 25% (●, ▲). Each point is the mean ± S.E.M. for four observations. The apparent first-order rate constants for [^{32}P]phosphorylation were (mean ± S.E.M., min^{-1}): fed, 12.5% KAP, 0.025 ± 0.001; fed, 25% KAP, 0.069 ± 0.001; starved, 12.5% KAP, 0.145 ± 0.008; starved, 25% KAP, 0.364 ± 0.025; $P < 0.01$ for effect of starvation and for differences between 12.5% and 25% KAP. The corresponding rate constants for ADP-dependent inactivation (progress curves not shown) were: fed, 25% KAP, 0.08 ± 0.005; starved, 12.5% KAP, 0.226 ± 0.072; starved, 25% KAP, 0.287 ± 0.014 (activity was not detected with fed, 12.5% KAP); $P < 0.01$ for effect of starvation. For other details see the Experimental section.

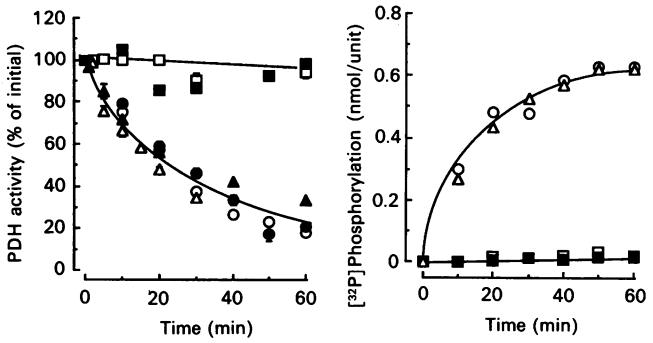

Fig. 2. [^{32}P]Phosphorylation and inactivation of *S. cerevisiae* PDH complex by KAP from livers of starved rats

KAP was prepared by method A (see the Experimental section). ATP-dependent inactivation and [^{32}P]phosphorylation by [γ-^{32}P]ATP were measured in separate experiments. Concentrations were: [γ-^{32}P]ATP, 0.5 mM (71 d.p.m./pmol); PDH complex (units/ml): ○, 0.5; ●, 1; △, □, 5; ▲, ■, 10. Incubations: no KAP (□, ■); plus KAP (○, ●, △, ▲). Results are means ± S.E.M. (error bars) for three observations (each point) for inactivation and means of duplicate observations (each point) for [^{32}P]phosphorylation. The apparent first-order rate constants for inactivation were (min^{-1}) 0.029 (○), 0.030 (●), 0.035 (△) and 0.029 (▲). For other details see the Experimental section.

Inactivation of KAP by FSBA

The evidence given in the preceding section suggested that KAP possesses PDH kinase activity, but does not wholly exclude the possibility that the pig heart PDHE1 and the yeast PDH

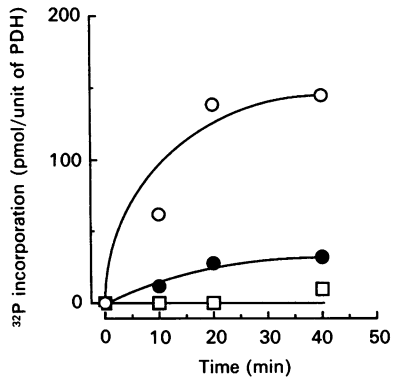

Fig. 3. [^{32}P]Phosphorylation of *S. cerevisiae* PDH complex by KAP from livers of fed (●) and 48 h-starved (○) rats

KAP was prepared by method A (see the Experimental section). Each point is the mean of duplicate observations. □, minus-KAP control. Concentrations were: [γ-^{32}P]ATP, 0.5 mM (40 d.p.m./pmol); *S. cerevisiae* PDH complex, 1 unit/ml. KAP added per ml was equivalent to 1.35 mg of mitochondrial protein. The activities of KAP with pig heart PDH complex were (units/ml): fed, 9.2; starved, 20. For other details see the Experimental section.

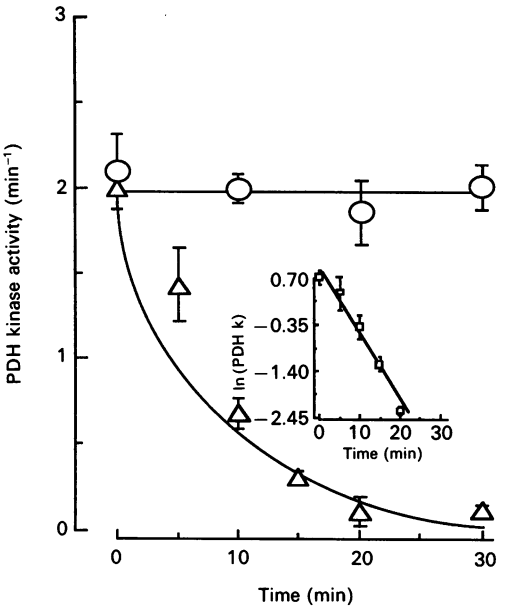

Fig. 4. Inactivation by 1 mM-FSBA of KAP prepared from livers of 48 h-starved rats

KAP was prepared by method B (see the Experimental section), and KAP activity was measured by its effect on the rate of ATP-dependent inactivation of purified pig heart PDH complex, expressed as the apparent first-order rate constant (±S.E.M.). The PDH kinase activity of pig heart PDH complex in the absence of KAP was < 0.1 min^{-1}. Each point is a single assay of KAP activity at the time shown. △, 1 mM-FSBA in 4% (v/v) dimethylformamide (DMF); ○, 4% DMF. The inset is a semi-logarithmic plot of the FSBA data in the main Figure, PDH k being PDH kinase activities. The apparent first-order rate constant for inactivation of KAP by FSBA was 0.108±0.018 min^{-1} ($t_{0.5}$ 6.4 min); $P < 0.01$ for significance of linear regression.

complex contain an inactive kinase which is activated on addition of KAP. For the *S. cerevisiae* complex, the absence of PDH kinase has only been demonstrated with highly purified complex, and the possibility that PDH kinase may be present in the yeast mitochondria has not apparently been excluded. Further evidence for the conclusion that KAP contains a PDH kinase was therefore sought by use of reagents which may inactivate protein kinases by forming adducts with the ATP-binding site. Two such reagents were studied, 8-azido-ATP [22] and FSBA [15–17]. Formation of adducts with 8-azido-ATP involves photolysis, and is only likely to be specific for ATP-binding sites if the binding constant is < 1 μM [22]. Significant inhibition of PDH kinase (pig heart

Table 1. Inactivation of (*a*) rat liver PDH kinase activator protein (KAP) and (*b*) intrinsic PDH kinase of rat liver PDH complex by FSBA and inhibition of inactivation by p[NH]ppA

Rat liver PDH complex (1.5 units/ml) and rat liver KAP prepared by method B (70 units/ml) were incubated for 60 min with additions as shown in 50 mM-potassium phosphate, pH 7.0. DTT was then added to 2 mM to quench FSBA, and samples were taken for assay of PDH kinase activity (PDH complex) or of KAP activity (measured by the stimulating effect of KAP on the PDH kinase activity of pig heart PDH complex) (column 3). Further assays were made after 18 h of dialysis against 50 mM-potassium phosphate/2 mM-DTT, pH 7.0 (column 4). PDH kinase activities were computed as apparent first-order rate constants for ATP-dependent inactivation. Results are means±S.E.M. for numbers of PDH kinase assays shown in parentheses. Within columns, $P < 0.01$, except *$P > 0.05$ for difference from FSBA alone and †$P > 0.05$ for difference from dimethylformamide (DMF) alone. For DMF alone, incubation with or without dialysis led to loss of activity of KAP ($P < 0.02$ for difference between columns 2 and 3, or 2 and 4), but activity of the intrinsic kinase of PDH complex was unaffected ($P > 0.05$). Dialysis increased activities after incubations with p[NH]ppA or with FSBA+p[NH]ppA ($P < 0.001$ for differences between columns 3 and 4).

Additions	PDH kinase activity (min^{-1}) for: Zero time	60 min incubation	60 min incubation, 18 h dialysis
(*a*) Rat liver KAP			
4% (v/v) DMF	3.33±0.20 (6)	2.70±0.03 (6)	1.76±0.16 (8)
1 mM-FSBA in DMF		0.18±0.08 (6)	0.33±0.03 (7)
1 mM-p[NH]ppA		1.29±0.04 (6)	2.05±0.02 (7)
1 mM-FSBA+1 mM-p[NH]ppA		0.14±0.11* (6)	0.92±0.10 (7)
(*b*) Rat liver PDH complex			
4% (v/v) DMF	1.79±0.10 (4)	1.86±0.08 (4)	2.12±0.08 (8)
1 mM-FSBA in DMF		0.35±0.04 (4)	0.61±0.03 (8)
1 mM-p[NH]ppA		0.78±0.09 (4)	3.78±0.10 (8)
1 mM-FSBA-p[NH]ppA		0.20±0.01 (4)	1.99±0.15† (8)

Table 2. Inactivation of rat liver KAP by PCMB and NEM

Rat liver KAP prepared by method C and freed of DTT (Sephadex G25) was incubated (2 min, 30 °C) either with 0.2 mM-PCMB, and after removal of PCMB (Sephadex G25) was concentrated with Centriflo cones, or with 2 mM-NEM, after which DTT was added to 5 mM and NEM was removed (Sephadex G25). §Incubated with 2 mM-DTT for 5 min at 30 °C. The activity of KAP was assayed through its effect on the activity of pig heart PDH kinase (measured as the apparent first-order rate constant for ATP-dependent inactivation). Each result is the mean ± S.E.M. for three or four observations, except where numbers of observations are shown in parentheses. For other details see the Experimental section. * $P < 0.001$ for effect of KAP; † $P < 0.01$ for effect of PCMB or of NEM; ‡ $P < 0.02$ for effect of DTT; ‖ activities assayed at the same protein concentration.

KAP (treatment)	PDH kinase activity (min^{-1})
None (control)	0.25 ± 0.014
Untreated‖	1.91 ± 0.064*
PCMB-treated (A)‖	0.27 ± 0.01†
(A) incubated with DTT§‖	2.50 ± 0.11*‡
None	0.18 ± 0.022 (20)
Untreated	1.09 ± 0.023* (35)
NEM-treated	0.26 ± 0.022† (36)

PDH complex + KAP) by 8-azido-ATP was only seen at concentrations in excess of 250 μM (ATP concentrations over the range 10–200 μM; K_m 12 μM), and experiments with this compound were discontinued. As shown in Fig. 4, 1 mM-FSBA inactivated KAP from livers of 48 h-starved rats by 95% in 30 min. The reaction was pseudo-first-order, with a rate constant of 0.1 ($t_{0.5}$ 6.5 min).

It seemed important to show further that FSBA inactivates the PDH kinase intrinsic to the PDH complex, and that ATP inhibits the effect of FSBA. For this purpose we have used PDH complex prepared from livers of starved rats (the intrinsic PDH kinase activity is some 20–40-fold greater than that of pig heart PDH complex); and we have used p[NH]ppA in place of ATP because it is a non-phosphorylating analogue (it is known that p[NH]ppA is a competitive inhibitor of intrinsic PDH kinase with respect to ATP; $K_i = 39$ μM [14]). These results are shown in Table 1. In these experiments 1 mM-FSBA produced 93% inhibition of rat liver KAP and 81% inhibition of PDH kinase intrinsic to rat liver PDH complex in 60 min (column 3, lines 1, 2, 5 and 6). Approx. 50% inhibition was given by 1 mM-p[NH]ppA (lines 1, 3, 5, 7), but calculation showed that this was due to inhibition by p[NH]ppA carried over into the kinase assay. Following dialysis (to remove p[NH]ppA), inhibition was no longer seen (indeed, some activation by p[NH]ppA was evident, but the reason for this is as yet unclear) (column 4, lines 1, 3, 5, 7). Inhibition of FSBA-induced inactivation by 1 mM-p[NH]ppA (approx. 50%) was apparent after dialysis (column 4, lines 2, 4, 6, 8), though not in undialysed samples (same lines in column 3). FSBA was quenched before PDH kinase assays with DTT [15]. Because PDH kinase and KAP are inactivated by thiol-reactive reagents ([11]; the following section), it was important to exclude a contribution by such a mechanism to inactivation of KAP by FSBA. It was found that protection of thiol groups with PCMB (removed later with DTT; see the following section) did not prevent inactivation by KAP by FSBA (results not shown).

Effect of thiol-reactive reagents on the activity of PDH kinase activator protein

Rat liver KAP (48 h-starved rats) was inactivated > 90% by 2 min of incubation at 30 °C with 0.2 mM-NEM (Table 2, column 4). A study of the time course showed that > 90% inactivation occurred within 1 min, with complete loss of activity occurring between 5 and 10 min (results not shown). In this time course, rapid removal of excess of NEM before assay of KAP was achieved by addition of DTT to 5 mM; no inactivation of KAP was observed on incubation for up to 10 min with 2 mM-NEM plus 5 mM-DTT (results not shown). There was no difference in the degree of inactivation over 2 min at 30 °C with 0.02 mM-, 0.2 mM- or 2 mM-NEM (results not shown). Inactivation of KAP by NEM was also apparent when PDH kinase activity was followed by [^{32}P]phosphorylation of PDH complex (results not shown). The results of experiments in Table 2 (column 2, lines 1–4) show that reaction of PCMB with thiol group(s) inactivated KAP and that the inactivation was completely reversed by incubation with DTT. (The reaction of PCMB with proteins is known to be confined to thiol groups, and re-activation by DTT further confirms this conclusion in respect of KAP.) KAP from liver mitochondria of fed rats was also inactivated by NEM and by PCMB (results not shown).

General discussion and conclusions

The results of the present study would appear to establish that preparations of PDH kinase activator protein (KAP) contain a PDH kinase. They do not entirely exclude the possibility that KAP preparations may also contain an activator of PDH kinase(s). The simplest interpretation of these findings is that mitochondria contain both free PDH kinase and PDH kinase bound to PDH complex. Alternatively, a fraction of PDH kinase may dissociate after solubilization of the complex and be separated during gel filtration or ultracentrifugation [though not during poly(ethylene glycol) precipitation], thus giving rise to the activity termed KAP. This could explain why the activities both of free PDH kinase (KAP) and of PDH kinase intrinsic to PDH complex are increased by starvation and are abolished by thiol-reactive reagents.

Free PDH kinase present in KAP preparations is quantitatively more important than bound PDH kinase intrinsic to the PDH complex (i.e. the PDH kinase which co-purifies with the complex). The PDH kinase activity of PDH complex separated from rat heart or rat liver mitochondrial extracts by gel filtration on Sephacryl S300 is only 15–30% of that of whole mitochondrial extract, and even higher kinase activities are obtained after recombining the Sephacryl S300 fractions [1,5]. The results of the present study have not addressed the question as to whether the PDH kinase in KAP and the PDH kinase fraction purified from the PDH complex by Stepp *et al.* [11] are identical proteins. Although substantial purification of KAP has been achieved (up to 2000-fold), several protein bands were still present on SDS gels (results not shown).

The PDH kinase activity that we have identified in KAP is a mitochondrial PDH kinase. The only other protein kinase identified in rat liver mitochondria is the branched-chain 2-oxoacid dehydrogenase kinase [23]. The PDH kinase in KAP is unlikely to possess branched-chain kinase activity. Gel filtration of rat liver mitochondrial extracts on Sephacryl S300 has no effect on the branched-chain kinase activity of the separated branched-chain complex [24], and precipitation of dehydrogenase complexes with poly(ethylene glycol) at pH < 7.0 separates branched-chain kinase from the complexes [23], but not the PDH kinase activity of KAP [5].

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