Volume 308, number 1, 83–86 FEBS 11397 August 1992

Purification and partial characterization of rat liver pyruvate dehydrogenase kinase activator protein (free pyruvate dehydrogenase kinase)

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Received 25 June 1992

Rat liver pyruvate dehydrogenase (PDH) kinase activator protein (KAP), a free PDH kinase readily separable from PDH complex and its intrinsic kinase, has been purified to apparent homogeneity from liver mitochondria of fed and 48-h starved rats. On SDS-PAGE an apparently single band of M_r 45 kDa was obtained. N-Terminal amino acid sequence analyses (8–10 cycles) confirmed the presence of a single peptide in each case. The specific activity of the purified KAP from 48-h starved rats (14,413 U/mg protein) was 4.5-fold greater than that from fed rats.

Pyruvate dehydrogenase kinase; Rat liver mitochondria; Starvation; N-terminal sequence; Specific activity

1. INTRODUCTION

Mitochondrial PDH complexes from mammalian tissues are inactivated by phosphorylation of the E1α component catalysed by a PDH kinase intrinsic to the complex [1]. Rat heart, liver and skeletal muscle mitochondria contain a protein (KAP), separated from rat PDH complex by gel filtration or ultracentrifugation, and which enhances the intrinsic PDH kinase activity of purified rat or pig PDH complexes [2–4]. Starvation or alloxan-diabetes increase the activities of PDH kinase and KAP in rat heart and liver mitochondria, 2- to 3-fold over 24–48 h [2–6]. In 24 h of culture of rat hepatocytes, cardiac myocytes and soleus muscle, *n*-octanoate, dibutyryl cAMP, and glucagon (hepatocytes only), increased activity of PDH kinase 2- to 3-fold [7–9], i.e. the effects of starvation or diabetes in vivo may be mediated by increased circulating FFA and tissue cAMP concentrations [9].

Several lines of evidence have suggested that KAP is a free PDH kinase which can be separated by gel filtration from the kinase intrinsic to PDH complex [10,11]. Thus KAP induces ^{32}P-phosphorylation and inactivation of pig or ox heart PDH.E1 free of PDH kinase [10,11] and of PDH complex from *Saccharomyces cerevisiae* which lacks PDH kinase [10]. KAP also catalyses ^{32}P-phosphorylation of a synthetic peptide substrate for PDH kinase [11]. KAP is inactivated by fluorosulphonyl-benzoyl-adenosine [10] which is known to form adducts with the ATP binding sites of protein kinases; and by thiol-reactive reagents [10] which are known to inactivate the intrinsic PDH kinase of bovine kidney PDH complex [12]. For convenience we shall continue to refer to this free PDH kinase as KAP.

In the present study KAP has been purified to apparent homogeneity from liver mitochondria of fed and starved rats and it is shown that starvation effects an approx. 4-fold increase in specific activity.

Abbreviations: PDH, pyruvate dehydrogenase; KAP, kinase activator protein; cAMP, adenosine 3′,5′ phosphate; PDH·E1, PDH·E2, E1 and E2 components of PDH complex.

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2. EXPERIMENTAL

Sources of rats and details of feeding were as in [7]. Biochemicals were from Sigma Chemical Co., Merck Ltd, or BCL; Matrex orange was from Amicon; Sephacryl S-300HR, Mono Q columns and FPLC apparatus were from Pharmacia; and protein standards for SDS-PAGE were from Bio-Rad. PDH complex was purified from pig hearts [13]. Rat liver mitochondria were isolated and extracts prepared by freezing and thawing as in [4], and clarified by centrifugation at 80,000 × *g* for 30 min.

PDH complex was assayed spectrophotometrically by the rate of NAD reduction as in [13]. Activity of KAP was assayed by its effect to increase the PDH kinase activity of pig heart PDH complex [4]. PDH kinase activity of pig heart PDH complex was assayed by the rate of ATP dependent inactivation using conditions as in [4] and computed as the apparent first-order rate constant. Protein was assayed with Coomassie blue employing bovine serum albumin as standard [14]. SDS-PAGE was as in [15] employing 0.75-mm gels at 5 mA for 16 h. N-terminal amino acid sequencing of purified KAP was carried out by Mr. Tony Willis of the MRC Immunochemistry Unit, University of Oxford and by Dr. K.S. Lilley of the Protein Sequencing Facility, Leicester University. After running on SDS-PAGE the gel was electroblotted to ProBlott membrane (Applied Biosystems, ABI) in a Bio-Rad mini Trans-Blot electrophoresis transfer cell. After staining with Coomassie blue the band was excised from

the membrane, inserted into an ABI B Blott cartridge, and sequenced using an ABI 470A Sequencer with an on-line PTH analyser [16].

3. RESULTS AND DISCUSSION

The starting point for the purification of KAP was a clarified extract of rat liver mitochondria (50 ml, 1.5 g protein) which had been incubated with digitonin (to remove lysosomes) and with carbonylcyanide *m*-chlorophenylhydrazone (to effect conversion of inactive to active PDH complex) as in [4]. The extract was in 20 mM potassium phosphate, 1 mM phenylmethanesulphonylfluoride, 0.3 mM *N*-α-*p*-tosyl-lysyl-chloromethyl-ketone, 1 mM benzamidine, 10 mM EGTA, 2 mM DTT, pH 7.25 (buffer A). The pH was adjusted to 6.5 with 10% (v/v) acetic acid at room temperature and KAP and PDH complex co-precipitated by addition of 0.1 vol. of 50% (w/v) poly(ethylene)glycol 6000 and taken up in 8 ml of buffer A. After gel filtration on a column of Sephacryl S300HR (450 ml), the higher specific activity KAP fractions were pooled (fractions 140–154 in Fig. 1) and KAP precipitated by addition of solid $(NH_4)_2SO_4$ to 40% saturation at 0°C. After stirring for 15 min and centrifugation the pellet was taken up in 3 ml of buffer A, added to 2.5 ml of Matrex orange equilibrated with buffer A, and incubated overnight (16 h) at 4°C with end-over-end rotation. The supernatant (containing the KAP) was separated by centrifugation and the pellet re-extracted once with 5 ml buffer A. The pooled supernatants were filtered on a Millex-GV filter unit (0.22 μ) (Millipore SA) and loaded onto a Mono Q FPLC column (1 ml) equilibrated with 20 mM potassium phosphate, 2 mM DTT, pH 7.25 (all flow rates 1 ml/min). After washing with 20 ml of the same buffer, stepwise elution was performed with 40 mM phosphate, 2 mM DTT, pH 7.25 (10 ml) followed by 95 mM phosphate, 2 mM DTT, pH 7.25 (10 ml). KAP was located by assay and the 1-ml fractions were pooled and concentrated approx. 10-fold in a Microsep concentrator (30K cut off) (Flowgen Instruments Ltd).

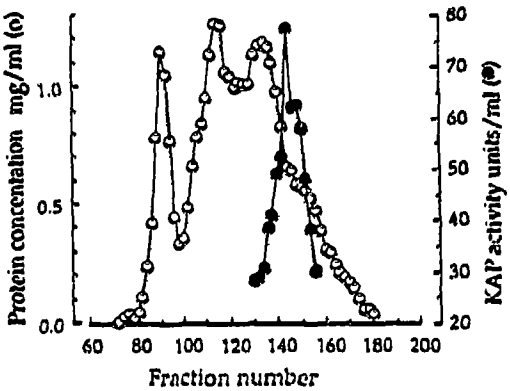

Fig. 1. Elution of KAP (free PDH kinase) from Sephacryl S300HR. PDH complex and KAP were co-precipitated from an extract of rat liver mitochondria (1.5 g protein) with 0.1 vol. of 50% (w/v) poly(ethylene)glycol 6000 and taken up in 8 ml of buffer A and applied to a 450-ml column of Sephacryl S300HR and eluted with the same buffer (2 ml fractions). Tubes 140–154 were routinely taken for subsequent steps in purification.

Table I

Yields and specific activities of KAP during purification from extracts of liver mitochondria of fed (F) or 48-h starved (S) rats

Step	Rat	Protein (mg)	KAP (U)[a]	Specific activity (U/mg)	Recovery (%)
Sephacryl S300 eluate	F	14.5	1,588	110	100
	S	16.7	5,758	345	100
Matrex orange supernatant	F	2.4	727	300	46
	S	1.8	2,071	1,146	36
Mono Q FPLC	F	0.11	341	3,217	21
	S	0.11	1,503	14,413	26

[a] One unit (U) of KAP is defined as that amount giving an apparent first-order rate constant in the PDH kinase assay (see section 2) of 1,386 min^{-1} ($t_{1/2}$ = 0.5 min). For details of individual steps in purification see text.

Typical purification profiles with Sephacryl S300HR concentrates as starting material are shown in Table I. KAP activity could not be assayed in mitochondrial extracts or poly(ethylene)glycol precipitates because of the presence of rat liver PDH complex and its intrinsic kinase. Yields of KAP from fed and starved rats through the two steps shown in Table I were similar. Yields were probably similar in the earlier stages of purification as the ratio (starved/fed) for total KAP units in the Sephacryl concentrate (3.62, Table I, column 4) does not differ substantially from that for the final product (4.48). SDS-PAGE of the final product (fed or starved) showed a single band of M_r 45 kDa (Fig. 2) whereas SDS-PAGE of Matrex orange supernatant (Fig. 2) showed additional bands of M_r 85, 65, 61, 53, 40, 38, and 29 kDa. Table I shows also that the specific activity of KAP from starved rats was 4.5-fold that from fed rats. In two other preparations not shown in Table I specific activities (U/mg) were 12,800 (starved) and 1,827 (fed). These preparations gave single bands on SDS-PAGE but were not subjected to N-terminal sequence analysis.

The results of N-terminal sequence analyses (8–10 cycles) of the major peak from Mono Q are shown in Table II for preparations from fed and starved rats (see Table I and Fig. 2 for other properties). The preparation from starved rats yielded only one residue at each cycle. The preparation from fed rats showed varying degrees of contamination with a second residue at seven of the eight cycles shown. The results indicate that KAP contains a single subunit of M_r 45 kDa by SDS-PAGE with the N-terminal sequence shown in Table II and that it has been purified to apparent homogeneity.

The intrinsic PDH kinase of bovine kidney PDH

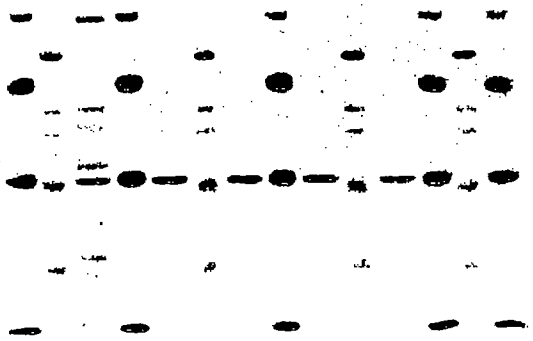

Fig. 2. SDS-PAGE of KAP (free PDH kinase) prepared from rat liver mitochondria of starved rats (specific activity 12,800 U/mg protein). Lanes A, D, H, L and N molecular weight markers, 97.4, 66.2, 45 and 31 kDa; lanes B, F, J and M purified pig heart PDH complex; lane C, Matrex orange supernatant; lanes E, G, I and K, highly purified KAP (Mono Q FPLC eluate) from 48-h starved rats. (KAP from fed rats is identical; not shown.)

Table II

N-terminal sequence analysis of KAP (free pyruvate dehydrogenase kinase, subunit M_r 45 kDa) purified from liver mitochondria of fed or 48-h starved rats as described in Table I (for typical SDS-PAGE see Fig. 2)

Cycle	Amino acid residue	Yield (pmol)		Other residues (pmol)	
		Fed	Starved	Fed	Starved
1	NH_2Lys	2.1	5.2	Ser (0.7)	-
2	Asn	4.9	8.0	Leu (0.5)	-
3	Ala	5.1	10.8	-	-
4	Ser	2.6	3.3	Gly (0.1)	-
5	Leu	2.4	2.8	Ala (0.4)	-
6	Ala	2.7	2.8	Pro (0.3)	-
7	Gly	2.7	4.7	Lys (0.1)	-
8	Ala	2.1	3.0	Tyr (0.5)	-
9	Ile	-	2.5		
10	Glu	-	1.8		
Total		23.6		2.6	

The results for KAP from fed rats are the means of three analyses on a single preparation and the results for starved rats are the means of two analyses on a single preparation, details of which are given in Table I. No other amino acid residues were detected in the starved preparation. In the fed preparation other residues were 11% of the major residues overall.

complex is a heterodimer composed of subunits of M_r (α) 48 and M_r (β) 45 kDa with kinase activity residing in the α-subunit [12]. The protein that we have purified and which has PDH kinase activity is apparently a single subunit and is therefore to be compared with the α-subunit of the bovine PDH kinase. The intrinsic kinase of rat PDH complex has yet to be fully characterized and it is not clear whether the apparently lower M_r of the free kinase subunit that we have isolated is due to species difference, or to differences in the procedures used in purification. Release of intrinsic PDH kinase from PDH complex involves chemical manipulation (incubation with DTT followed by treatment with *p*-hydroxymercuriphenyl sulphonate [12]) whereas free kinase was separated by gel filtration (present study).

A lower subunit M_r could be the result of proteolysis, but the precautions against proteolysis during our preparation of rat liver KAP (i.e. free PDH kinase) were at least as stringent as those utilised in the preparation of bovine kidney PDH kinase in [12]. The rat liver mitochondrial fraction was freed of lysosomes by treatment with digitonin and washing with KCl medium as in [4]; and protease inhibitors (see section 2) were present throughout disruption of mitochondria and all subsequent steps up to mono Q FPLC. It is well established that PDH complex, which is sensitive to proteolysis, is stable during incubation of mitochondrial extracts prepared by this method [4]. Bovine kidney PDH kinase α-subunit is sensitive to chymotrypsin, which inactivates the kinase [12], but there is no reason to suspect loss of activity in the course of purification of free rat liver PDH kinase in the present study. The specific activity of the intrinsic PDH kinase purified from bovine kidney complex in [12] was 130 mU/mg protein where 1 U gives 1 μmol ^{32}P-phosphorylation of bovine PDH E1/E2 subcomplex/min. The computed equivalent specific activities of the rat liver PDH kinases in the present study (Table I) and using the same units were: fed, 276 U/mg; and starved, 1235 U/mg (the calculation is based on an incorporation of approx. 0.3 nmol of [^{32}P]phosphate/U of pig heart PDH complex inactivated [17]).

The present study has shown further that starvation increases the specific activity of highly purified free rat liver PDH kinase approx. 4-fold thus confirming conclusions drawn from studies with relatively crude KAP separated from PDH complex by gel filtration and assayed with purified pig heart PDH complex [4] or with pig heart PDH E1 or *S. cerevisiae* complex [10]. Failure to demonstrate such an increase in specific activity in an earlier study in which KAP was separated from PDH complex by ultracentrifugation [2] is attributed to the presence of low molecular weight inhibitors of PDH kinase (e.g. ADP, thiamin pyrophosphate) which are removed when KAP is prepared by gel filtration. The mechanism responsible for this increase in PDH kinase specific activity with starvation has yet to be determined.

Acknowledgements: These studies were supported by grants from the British Heart Foundation and the British Diabetic Association.

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