Phosphoenolpyruvate-dependent protein kinase activity in rat skeletal muscle

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Phosphoenolpyruvate-dependent protein kinase activity has been demonstrated in the soluble fraction of rat skeletal muscle. The reaction was not due to the formation of ATP in the incubation mixture. Cyclic AMP, calcium, ATP and a number of phosphate acceptor proteins did not stimulate the reaction. One ^{32}P-labelled protein (M_r 25000) was observed on SDS gels. The phosphorylated protein contained acid stable phosphoserine as a major phosphorylated amino acid. The phosphorylation reaction in crude extracts was not directly proportional to the amount of protein, but typical of a two-component system; i.e., kinase and substrate. The chromatography of soluble proteins on Ultrogel AcA44 separated the phosphate acceptor protein(s) from the phosphoenolpyruvate-dependent protein kinase activity.

Protein kinase *Phosphoenolpyruvate* *Skeletal muscle* *Phosphoprotein*
Phosphorylation *Regultation*

1. INTRODUCTION

There is one well-characterized phosphoenolpyruvate-dependent protein kinase, and it is enzyme I of the bacterial phosphoenolpyruvate: sugar phosphotransferase system (PTS). The PTS is found in a number of bacterial species, and has been well described in both *Escherichia coli* and *Salmonella typhimurium* [1,2]. The PTS is responsible for the concomitant phosphorylation and translocation of a number of hexoses and hexitols. Moreover, it has been implicated in a number of regulatory phenomena such as catabolite repression, inducer exclusion and catabolite inhibition [2]. Enzyme I is a soluble component of this transport system that is known to phosphorylate two soluble phosphocarrier proteins, histidine containing protein (HPr) and fructose-induced HPr-like protein (FPr) [3] which are also part of the PTS. It has been postulated [2] that the regulatory interactions of the PTS such as adenylate cyclase regulation [4] may be due to phosphorylation events dependent upon enzyme I and phosphoenolpyruvate (PEP). The products of enzyme I phosphorylation contain 1-P-histidine [5] which is an acid-labile derivative [6].

The covalent modification of proteins by phosphorylation–dephosphorylation is one of the major mechanisms by which intracellular events in mammalian tissues are controlled by nervous and hormonal stimuli [7,8]. In the vast majority of phosphorylation reactions, ATP is the most preferred phosphate donor although other nucleoside triphosphates like GTP, UTP or ITP are known to donate phosphate at least in vitro [8]. It has not been shown whether any high energy compound other than these nucleotides can donate phosphate in mammalian phosphorylation reactions. Here, we provide evidence for the presence of a PEP-dependent protein kinase activity in the soluble fraction of skeletal muscle of rat. The phosphorylated product contained acid stable phosphoserine as the major phosphorylated amino acid.

2. MATERIALS AND METHODS

[^{32}P]PEP was prepared as described [9]. The PTS proteins enzyme I and HPr were prepared as has been described [10] from *E. coli* P650. The fac-

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tor III^{glc} preparation from *E. coli* P650 was based upon procedures developed in *S. typhimurium* [11]. Phosphoserine, phosphothreonine, histone, phosvitin, protamine, casein, 2-(*N*-morpholino)-ethane sulfonic acid (Mes), benzamidine, and ATP were from Sigma. Ultrogel AcA44 was obtained from Fisher Scientific Co. All other reagents were of analytical grade.

2.1. *Preparation of rat tissue extracts*

Rats were decapitated and skeletal muscle was immediately removed, homogenized in 4 vol. 20 mM glycylglycine (pH 7.4), 10% glycerol, 15 mM β-mercaptoethanol and 0.5 mM benzamidine. The homogenates were centrifuged at 100000 × *g* for 1 h. The clear supernatants were dialyzed overnight against the same buffer for use in these studies.

2.2. *Protein kinase activity*

The protein kinase activity was measured in incubation mixtures that contained 25 mM Mes (pH 6.9), 0.1 mM [^{32}P]PEP (2–10 × 10^5 cpm/nmol), 5 mM $MgCl_2$, 0.25 mM EGTA and crude, dialyzed, soluble proteins of skeletal muscle in 0.05 ml final vol. Any alterations in this mixture are indicated in the figure legends or in the text. The incubation was for 10 min at 30°C. The radioactive protein was precipitated on a Whatman 31 ET paper, washed and counted for radioactivity as in [12]. One unit of enzyme activity was defined as the amount of enzyme that incorporated 1 pmol ^{32}P from [^{32}P]PEP/min.

2.3. *Phosphoamino acid identification*

Crude soluble proteins (about 0.2 mg) were incubated for 10 min with 0.1 mM [^{32}P]PEP as described for protein kinase activity measurement. The proteins were precipitated and washed using cold trichloroacetic acid, and the labelled protein was hydrolyzed, and chromatographed as in [13]. Phosphoserine and phosphothreonine at 1 mg/ml were used as internal standards.

2.4. *Analytical methods*

Protein was determined as in [14] using bovine serum albumin as standard. PEP was separated from ATP on DEAE-cellulose paper (Whatman DE 81) as in [9].

3. RESULTS AND DISCUSSION

When the crude soluble proteins from rat skeletal muscle were incubated with [^{32}P]PEP and Mg^{2+}, the resulting acid-stable phosphorylation of proteins detected by trichloroacetic acid precipitation showed both protein concentration and time dependency (fig.1). Because the crude soluble proteins provided both the substrate and kinase, the increasing amount of phosphorylation was not directly proportional to the protein concentration in the assay (fig.1A). Within the range of protein tested, reactions were linear for at least 10 min incubation time (fig.1B), and therefore, assays employing crude soluble proteins were carried out using about 0.2 mg protein with a 10 min incubation time. The K_m for PEP was 0.11 mM which is a physiological concentration.

It was of prime concern whether these phosphorylations were due to [γ-^{32}P]ATP formed by pyruvate kinase from [^{32}P]PEP during the incubation despite the dialysis to remove endogenous nucleotides. For this reason, incubation samples taken at various times were chromatographed on a DEAE-cellulose paper [9] for the separation of ATP from PEP. There was no detectable [γ-^{32}P]ATP formed during the reaction and almost the entire radioactivity was associated with PEP and a little with P_i. Further evidence against ATP involvement is

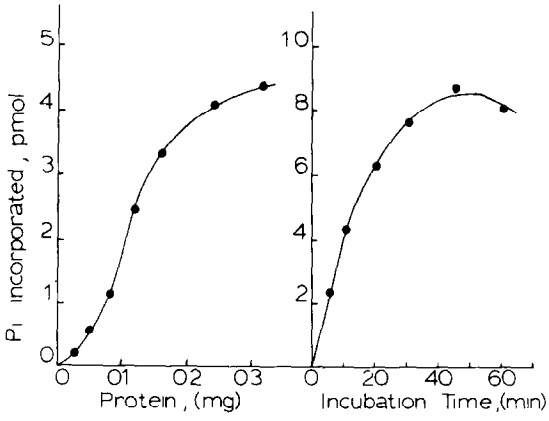

Fig.1. Phosphorylations produced by [^{32}P]phosphoenolpyruvate: (A) incubations were carried out as in section 2 with increasing amounts of soluble proteins from rat skeletal muscle for 10 min; (B) the incubations were carried out with 0.12 mg rat skeletal muscle proteins, and samples taken at the times shown.

Table 1

Effect of cyclic AMP, NaF, calcium, ATP and protein substrates on PEP-dependent protein kinase activity in rat skeletal muscle crude extract

Additions	Skeletal muscle	
	Units/mg protein	Relative %
Expt.1		
None	5.8	100
Cyclic AMP (10 μM)	5.8	100
Histone (0.2 mg)	2.6	45
Histone (0.2 mg) + cyclic AMP (10 μM)	2.7	47
NaF (12.5 mM)	3.9	67
$CaCl_2$ (125 μM)	4.8	83
ATP (0.1 mM)	5.3	92
Expt.2		
None	6.9	100
Albumin (0.2 mg)	7.0	101
Casein (0.2 mg)	3.8	55
Protamine (0.2 mg)	2.4	35
Phosvitin (0.2 mg)	4.1	59

found in table 1. Non-radioactive ATP added to the incubations did not inhibit PEP-dependent phosphorylations of proteins. The results in table 1 also show that PEP did not phosphorylate a variety of protein substrates usually used for the detection of ATP-dependent protein kinase activity. Histone was not phosphorylated both in the absence or presence of cyclic AMP. In fact, most of these protein substrates inhibited the [^{32}P]PEP-dependent phosphorylation of rat skeletal muscle proteins. Cyclic AMP alone had no effect. NaF and $CaCl_2$ also inhibited the phosphorylation of skeletal muscle proteins.

The phosphorylated skeletal muscle samples were resolved by SDS–PAGE, and the resulting autoradiograph showed one major phosphoprotein band at about M_r 25000 (fig.2). The occurrence of just one major phosphoprotein in skeletal muscle further rules out the involvement of ATP in these reactions. In the presence of ATP, a host of proteins were phosphorylated under these conditions (unpublished). The major phosphoamino acid produced has been identified as phosphoserine. With long exposure of the plate, a faint spot corresponding to phosphothreonine was also found in the muscle samples (2 out of 3 times), but in much lower amounts than phosphoserine. As enzyme I of the PTS produces 1-P-histidine in its products [5] this eukaryotic protein kinase does not appear to be similar. Enzyme I did not produce any detectable phosphorylation when added to these eukaryotic proteins with [^{32}P]PEP (unpublished).

The rate of phosphorylation of rat skeletal muscle soluble proteins in the presence of [^{32}P]PEP was about 6–10 pmol.mg protein^{-1}.min^{-1}. Under these conditions the total incorporation of phosphate was, however, over 200–250 pmol/mg protein. Assuming that the phosphorylated protein had M_r 25000–50000 and was as high as 0.1% of the total soluble proteins of skeletal muscle, this level of phosphorylation would be sufficient to incorporate 2–3 mol phosphate/mol protein.

To gain more information on the phosphate acceptor protein substrate and the kinase activity in rat skeletal muscle soluble proteins, attempts were made to separate these two components chromatographically by gel filtration using Ultrogel AcA44. As a first step, soluble proteins were phosphorylated in the presence of [^{32}P]PEP and chromatographed on this column. A single radioactive protein peak was observed and the bulk of the radioactivity eluted later as [^{32}P]PEP and $^{32}P_i$ (fig.3). The approximate M_r of this radioactive protein peak was 50000 which may be a dimer since the M_r of phosphorylated protein determined by SDS–PAGE and autoradiography was 25000. Further studies are needed to confirm this point.

It was of further interest to know whether the protein component capable of being phosphorylated could be resolved free of the PEP-dependent kinase activity for use as substrate in future experiments. For this purpose, non-phosphorylated rat muscle crude soluble proteins were fractionated using the same column and fractions (38–47) were pooled (fig.3). Catalytic amount of crude extract (0.04 mg protein) was used as a source of the kinase activity. The pooled fraction could not be used as a source of phosphate-acceptor protein substrate since our results indicated that this fraction also contained a significant amount of the kinase activity.

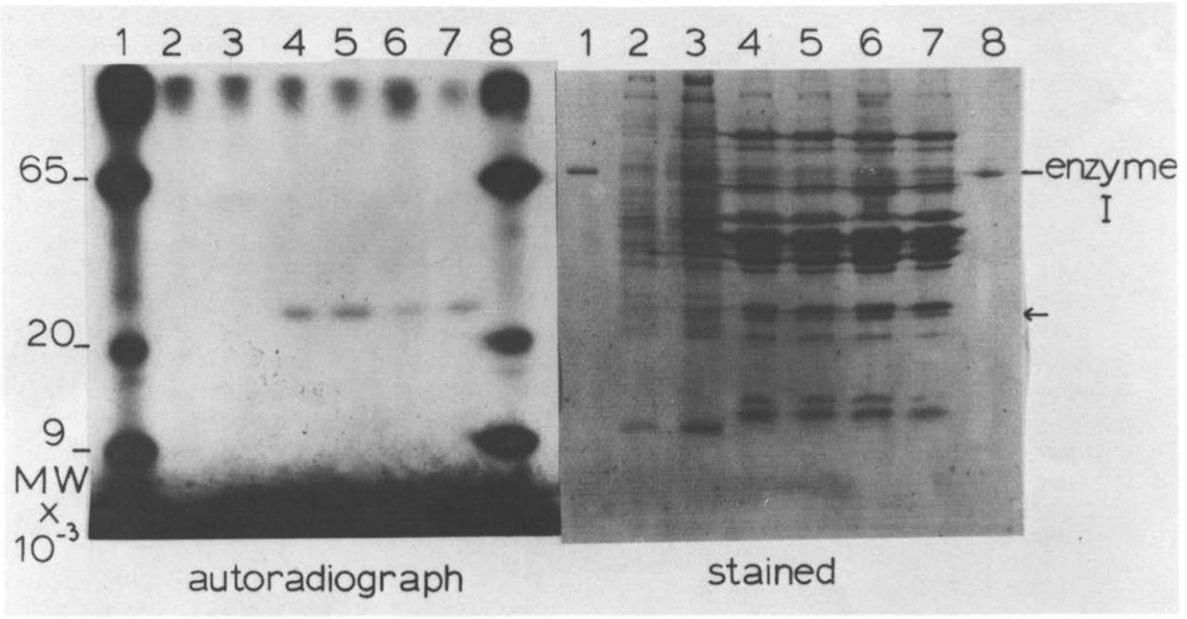

Fig.2. SDS–PAGE and autoradiography. This was carried out using a Biorad Protean apparatus with 0.75 mm slab gels that were prepared according to [15]. The samples and gels were treated and run such that the resolution of proteins with either acid-stable or acid-labile phosphorylations can be obtained (unpublished). Samples of soluble proteins were incubated at room temperature with sample buffer [15] at pH 8.0 for 20–30 min, a period of time taken to prepare and load samples. The SDS–PAGE was run in a precooled (10°C) apparatus at 20 mA/gel for 4–5 h. The gels were then frozen quickly using a dry ice–ethanol bath, and the autoradiographs obtained at −70°C [16]. The [^{32}P]phosphorylated PTS proteins enzyme I (M_r 65000), factor III^{glc} (M_r 20000) and HPr (M_r 9000) were used as M_r-markers for the autoradiograph (lanes 1,8); lanes 2,3 were liver samples; lanes 4–7 were rat skeletal muscle samples (78 μg). The phosphorylation conditions for lanes 4–7 were as follows: (4) no addition; (5) with cyclic AMP; (6) with NaF; (7) with $CaCl_2$. The other details were similar to conditions described in table 1.

It was unlikely that the phosphate acceptor protein substrate and the kinase had identical M_r and, therefore, eluted in the same fractions from the column. If these two proteins had somewhat different M_r-values then only a part of the peak of the substrate would overlap with the peak of the kinase activity. Under these conditions, one should be able to separate the ascending part of one protein peak from the descending part of the second protein peak. For this purpose, crude extract was chromatographed by gel filtration and various fractions were pooled into 5 different batches (pools A–E; fig.3). Pool C was the position where phosphorylated protein eluted. These five pools were concentrated 25-fold by ultrafiltration (Amicon Pm 10) and phosphorylated with [^{32}P]PEP in the absence or presence of crude extract. In the absence of crude extract, the maximum phosphorylation was obtained with pool C (table 2). However, in the presence of crude extract as a source of kinase activity, maximum phosphorylation was observed with pool B. The net maximum increase in phosphorylation by crude extract was also obtained with pool B. These results indicated that although pool C contained the maximum amount of substrate protein, pool B contained the protein substrate with the least contamination of the kinase activity.

In another experiment pool B was used as a source of substrate with pool E, as a source of kinase activity. Fig.4 shows the amount of phosphorylation when proteins from one of these pools were kept constant and proteins from the other pool varied. As shown, an increase in the amount of pool E did not increase the total phosphorylation of proteins from pool B. The

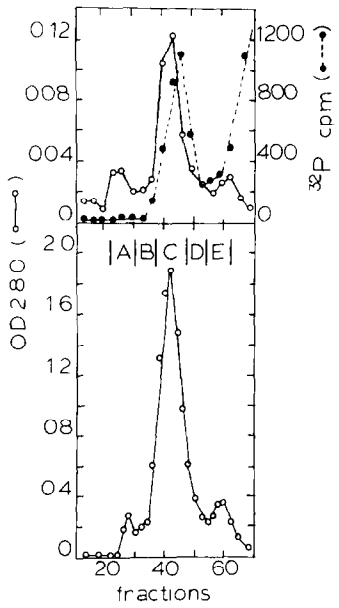

Fig.3. Chromatography of rat skeletal muscle crude extracts on Ultrogel AcA44. Upper: Crude extract (8 mg) was phosphorylated with [^{32}P]PEP as in section 2 and applied to a column of Ultrogel AcA44 (2.5 × 60 cm) pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.5), 0.5 mM EDTA, 0.2 mM dithioerythreitol, and 50 mM NaCl. The column was run with a flow rate of 30 ml/h and fractions of 80 drops (app. 5 ml) were collected; A_{280} (○---○); ^{32}P cpm (●---●). Lower: Rat skeletal muscle crude extract (160 mg) without any prior phosphorylation was applied to the same column and eluted as above. Other conditions were similar to those described for the above column. Fractions containing proteins were pooled in 5 separate pools: (A) fractions 22–30; (B) 31–37; (C) 38–47; (D) 48–54; (E) 55–62. Each pool was concentrated 25-fold with Amicon PM-10 membrane before using for experimental studies.

level of phosphorylation was totally dependent on the amount of protein used from the pool B in the assay. This further confirmed that pool B contained protein substrate(s) whereas pool E provided the kinase activity. These results, however, do not rule out the possibility that kinase activity may be made up of two non-identical subunits, of which one is in pool B and the second is in pool E. Although this is an unlikely possibility, it has to be examined later after obtaining the purified PEP-dependent protein kinase.

A further concern was that the phosphoprotein formed is the true phosphorylated protein and is not an enzyme intermediate. Although this possibility can not be completely ruled out at this

Table 2

Phosphorylation of concentrated pooled fractions obtained from Ultrogel AcA44 column in the absence and presence of 0.04 mg rat skeletal muscle crude extract

Pools	Phosphorylation (cpm incorporated)		
	Without crude extract	With crude extract	Phosphorylation increased by crude extract
Crude extract alone	–	518	–
A[a]	760	1089	Nil
B	551	5182	4113
C	2960	4841	1363
D	1545	2982	919
E	844	1590	228

[a] Various pooled fractions were collected and concentrated as in the legend of fig.3

Assay conditions were the same as in section 2, except that 0.03 ml concentrated pool fractions were used in the assay. Specific activity of [^{32}P]PEP was 180 cpm/pmol

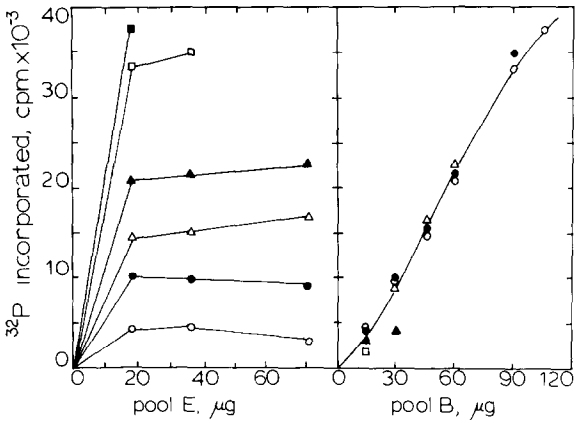

Fig.4. Phosphorylation of proteins using concentrated pools B and E obtained from Ultrogel AcA44 column. Left: Protein levels of pool E were varied in phosphorylation reactions using several fixed amounts of proteins from pool B. Pool B protein concentrations were: 15 μg (○); 30 μg (●); 45 μg (△); 60 μg (▲); 90 μg (□); and 105 μg (■). Right: Protein levels of pool B were varied in phosphorylation reactions using several fixed amounts of proteins from pool E. Pool E protein concentrations were: 17 μg (○); 35 μg (●); 70 μg (△); 105 μg (▲); 120 μg (□). These fractions (pools B,E) were obtained and concentrated as in fig.3. Conditions for phosphorylation reactions were as described in section 2 except that the proteins from pool B and E were varied as indicated above.

time with the use of crude extracts or the partially purified proteins, the following 3 results are against an enzyme intermediate:

(1) An enzyme intermediate would not show a time-dependent phosphorylation;
(2) The incubation of [^{32}P]phosphoproteins pooled from Ultrogel AcA44 column (fig.3) could not be dephosphorylated in the presence of 0.1–40 mM pyruvate at 37°C up to 20 min incubation;
(3) The phosphorylation of an enzyme intermediate would not usually require two separate proteins as observed with different pools from gel filtration column (table 2 and fig.4).

Furthermore, if these pools (B,E) were to contain non-identical subunits of a protein capable of forming a complex with PEP, one should not ordinarily observe an increased phosphorylation on addition of crude extract to the individual pools.

All these experiments described in this study indicate the presence of a PEP-dependent protein kinase in the soluble fraction of rat skeletal muscle. It represents the first report of a protein phosphate donor other than nucleotides like ATP. Further work is in progress to purify and characterize the PEP-dependent protein kinase as well as the substrate(s) for this kinase from rat skeletal muscle.

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