Phosphocreatine-dependent protein phosphorylation in rat skeletal muscle

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Phosphocreatine (PCr) was found to alter the phosphorylation state of two proteins of apparent molecular masses 18 and 29 kDa in dialysed cell-free extracts of rat skeletal muscle in the presence of [γ - 32 P]ATP. The 29 kDa protein was identified as phosphoglycerate mutase (PGM), phosphorylated at the active-site histidine residue by 2,3-bisphosphoglycerate (2,3-biPG). 2,3-biPG labelling from [γ - 32 P]ATP occurred through the concerted action of phosphoglycerate kinase and 2,3-bisphosphoglycerate mutase. PCr-dependent labelling, which required creatine kinase, resulted from a shift in the phosphoglycerate kinase equilibrium towards 1,3-bisphosphoglycerate (1,3-biPG) synthesis, ultimately resulting in an increase in available [2- 32 P]2,3-biPG. The maximal catalytic activity of PGM was unaffected by PCr. The 18 kDa protein was transiently phosphorylated at a histidine residue, probably by 1,3-biPG. No proteins of this monomeric molecular mass are known to bind 1,3-biPG, suggesting that the 18 kDa protein is an undescribed phosphoenzyme intermediate. Previous observations of 2- and 3-phosphoglycerate-dependent protein phosphorylation in cytosolic extracts [Ueda & Plagens (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1229–1233; Pek, Usami, Bilir, Fischer-Bovenkerk & Ueda (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4294–4298], attributed to the action of novel kinases, are likely to represent phosphoenzyme intermediates labelled by bisphosphorylated metabolites in a similar manner.

INTRODUCTION

Mature skeletal muscle, although post-mitotic, remains adaptive and can alter its phenotype in response to changing physiological demands. Evidence from a variety of studies suggests that skeletal muscle phenotype is primarily determined by input from the motor nerve [reviewed by Salmons & Henriksson (1981) and Pette & Vrbova (1985)]; however, the nature of the intracellular signals which mediate this response are unknown. A number of studies (Fitch *et al.*, 1975; Annesley & Walker, 1980; Mainwood *et al.*, 1982; Mainwood & De Zepetnek, 1985; Shoubridge *et al.*, 1985; Meyer *et al.*, 1986; Shoubridge & Radda, 1987) have shown that chronic depletion of phosphocreatine (PCr) is sufficient to cause phenotypic alteration in rodent skeletal muscles, raising the possibility that metabolic signals are involved in phenotypic determination. PCr, the cellular energy buffer, can be depleted by 90% by feeding rats with the creatine substrate analogue β -guanidinopropionic acid (GPA) (Fitch *et al.*, 1974). GPA accumulates in muscle as the phosphorylated form of GPA (PGPA), but cannot substitute for PCr because PGPA is a poor substrate for creatine kinase (EC 2.7.3.2) (Chevli & Fitch, 1979).

GPA feeding produces several phenotypic alterations in rat skeletal muscles. (1) The maximal activities of key glycolytic enzymes are down-regulated, whereas those of aerobic enzymes are unchanged or slightly increased (Shoubridge *et al.*, 1985). (2) Muscle glycogen content is increased 2-fold, but the rate of glycogenolysis is decreased during low-frequency stimulation (Shoubridge *et al.*, 1985; Meyer *et al.*, 1986). (3) Mechanical performance (twitch and tetanic tension) is altered, depending on the duration and intensity of muscle work (Shoubridge & Radda, 1987; Meyer *et al.*, 1986). (4) The staircase effect is abolished (Fitch *et al.*, 1975; Mainwood *et al.*, 1982; Meyer *et al.*, 1986), and post-tetanic twitch potentiation is reversed (Mainwood & De Zepetnek, 1985). (5) Expression of the slow isoforms of

myosin heavy and light chains is up-regulated (Moerland *et al.*, 1989; E. A. Shoubridge, unpublished work). Depletion of PCr with cyclocreatine, another creatine analogue, has been reported to suppress adrenaline-induced glycogenolysis in mouse muscles with an intact circulation (Annesley & Walker, 1980). The relationship between the decrease in PCr, the accumulation of a non-functional phospho-analogue and phenotypic alteration is unknown; however, the data suggest that changes in PCr/phospho-analogue pools can have profound effects on metabolic control mechanisms. Reversible protein phosphorylation plays a major regulatory role in the control of glycogenolysis/glyconeogenesis (reviewed by Gutman, 1985), isometric twitch tension development (Moore & Stull, 1984; Palmer & Moore, 1989) and post-tetanic twitch potentiation (Manning & Stull, 1982). On this basis, we hypothesized that PCr might play a role in modifying protein phosphorylation patterns. Modulation of protein phosphorylation by the glycolytic metabolites 2- and 3-phosphoglycerate (2-PG, 3-PG) has been reported in cell-free extracts from a variety of mammalian tissues (Ueda & Plagens, 1987; Pek *et al.*, 1990). It has been suggested that these phosphorylation events result from the action of novel kinases, and that they may be important in glycolytic regulation (Ueda & Plagens, 1987; Pek *et al.*, 1990).

Here we demonstrate PCr-dependent phosphorylation of two cytosolic proteins of 18 and 29 kDa in dialysed cell-free extracts of skeletal muscle. The latter of these two proteins was identified as the glycolytic enzyme phosphoglycerate mutase (PGM) (EC 5.4.2.1), phosphorylated by 2,3-bisphosphoglycerate (2,3-biPG) at the enzyme active-site histidine residue. The label transfer from [γ - 32 P]ATP to [2- 32 P]2,3-biPG occurred via phosphoglycerate kinase (EC 2.7.2.3) and 2,3-biPG mutase (EC 5.4.2.4). PCr, in the presence of creatine kinase, affected the phosphorylation state of PGM by shifting the equilibrium of phosphoglycerate kinase in the direction of 1,3-bisphospho-

Abbreviations used: PCr, phosphocreatine; GPA, β -guanidinopropionic acid; PGPA, phosphorylated GPA; PGM, phosphoglycerate mutase; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; 1,3-biPG, 1,3-bisphosphoglycerate; 2,3-biPG, 2,3-bisphosphoglycerate; cAMP, cyclic AMP; IBMX, isobutylmethylxanthine; WGAS, white gastrocnemius; G-1,6-biP, glucose 1,6-bisphosphate; F-2,6-biP, fructose 2,6-bisphosphate.

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glycerate (1,3-biPG) synthesis, resulting in an increase in available [^{32}P]2,3-biPG. The 18 kDa protein, which was not identified, was also phosphorylated on a histidine residue, probably by 1,3-biPG. The presence of novel metabolite-dependent kinases need not be invoked to explain phosphorylation events which depend on the presence of glycolytic intermediates in cell-free cytosolic extracts; most are probably phosphoenzyme intermediates, labelled by bisphosphorylated metabolites.

MATERIALS AND METHODS

Materials

PGM, phosphoglycerate kinase, enolase (EC 4.2.1.11), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40), the disodium salt of PCr, the pentacycloammonium salt of 2,3-biPG, the trisodium salt of 3-PG, the free acid of cyclic AMP (cAMP), calmodulin and aprotinin were from Boehringer Mannheim. Isobutylmethylxanthine (IBMX) and phosphatidylserine were obtained from Sigma Chemical Co., and [γ - ^{32}P]ATP (sp. radioactivity 4500 Ci/mmol) was from ICN Biomedicals. All other chemicals used in this study were of analytical grade or of the highest purity available. PCr was purified by ion-exchange chromatography (Fitch *et al.*, 1979) to remove impurities which have been shown to inhibit pyruvate kinase and phosphofructokinase (EC 2.7.1.11). To check the effectiveness of this procedure, ^{31}P -n.m.r. (at 162 MHz) and ^1H -n.m.r. (at 400 MHz) spectra of purified and unpurified PCr were obtained with a Varian VXR-400 spectrometer. The ^{31}P -n.m.r. spectra of purified and unpurified PCr showed a single resonance. Two unassigned resonances in the ^1H -n.m.r. spectrum of unpurified PCr were absent from the ^1H -n.m.r. spectrum of purified PCr (results not shown).

Animals

Male Wistar rats (200–300 g) were obtained from Charles River and were allowed free access to rat chow and water.

Sample preparation

Rats were anaesthetized with diethyl ether or by intraperitoneal injection of sodium pentobarbital (60 mg/kg) 10 min before being killed by cervical dislocation. The superficial white gastrocnemius (WGAS) muscle (which is composed entirely of type IIB fibres), soleus muscle, liver and heart were dissected, weighed and homogenized in 9 vol. of ice-cold extraction buffer [10 mM-Tris/HCl, pH 7.4, 2 mM-EDTA (free acid), 1 mM-dithiothreitol, 0.2 mM-phenylmethanesulphonyl fluoride and 200 mM-aprotinin] (Walaas *et al.*, 1983) on a Kinematica CH-6010 Polytron. The resulting homogenate was spun at 1850 g for 10 min to remove cellular debris, and the supernatant was collected and spun at 31 300 g for 1 h at 4 °C. The supernatant (1 ml) was dialysed in a Spectra-por3 membrane (Fisher) with a molecular-mass cut-off of 3.5 kDa for 16 h at 4 °C in 1 litre of extraction buffer.

Phosphorylation experiments

The dialysed supernatant was used in protein-phosphorylation assays as described in Walaas *et al.* (1983). Experiments were performed at 37 °C in an incubation buffer (25 mM-Tris/HCl, pH 7.4, 6 mM-MgSO₄, 1 mM-EDTA, 1 mM-EGTA and 1 mM-dithiothreitol). The supernatant (40 μl ; protein content approx. 150 μg , measured by the Lowry method as modified by Kresze, 1984) was added to the incubation buffer with or without (i) PCr, (ii) effectors of protein kinases or phosphatases, or (iii) dialysed purified commercial enzyme(s) (final volume 90 μl) and incubated for 90 s. Protein phosphorylation was started by addition of

9.5 μl of 20 μM -ATP mixed with 0.5 μl of [γ - ^{32}P]ATP (sp. radioactivity 22.5 Ci/mmol) and allowed to proceed for 30 s. The reaction was stopped by addition of 50 μl of stop solution (186 mM-Tris/HCl, pH 6.7, 15% glycerol, 7% SDS, 0.5 mg of Bromophenol Blue/ml and 6% mercaptoethanol) (Ueda & Plagens, 1987). The reaction mixture was heated to 90 °C for 2 min, allowed to cool to room temperature, and 10–20 μl samples (10–20 μg of protein) were separated by SDS/PAGE on 12.5% or 14%-acrylamide 1 mm-thick slab gels by the method of Laemmli (1970). Gels were fixed in 30% methanol/10% acetic acid for 1 h. In some experiments the gel was quickly stained for 10 min and subsequently destained for 20 min. This fast staining/destaining protocol was used to permit protein detection without discernible loss of labelling. Gels were then dried and exposed to Kodak X-OMAT film with an intensifying screen.

Identity of the phosphorylated amino acids

To determine the identity of the phosphorylated amino acid residues, SDS gels containing the phosphorylated proteins were subjected to acid or base hydrolysis. After electrophoresis, the gels were soaked in double-distilled deionized water for 30 min and then subjected to either 5% HClO₄ acid for 15 min at 95 °C or 1 M-NaOH for 1 h at 60 °C. Gels were then rinsed in double-distilled deionized water, dried and subjected to autoradiography as described above. Treated gels were compared with untreated control gels containing the same samples.

Muscle supernatant fractionation

WGAS muscle supernatant (undialysed) was separated on a column (2 cm $\times$ 87 cm) of Sephadex G-100 (Pharmacia), which was swollen in and eluted with extraction buffer. The column was calibrated with the following proteins (used as molecular-mass standards): cytochrome *c* (12.3 kDa), phosphoglycerate kinase (47 kDa), creatine kinase (86 kDa) and glyceraldehyde-3-phosphate dehydrogenase (142 kDa). Column fractions were collected, pooled and concentrated by using Centricon 10 or 30 filters (Amicon). The approximate molecular-mass ranges covered by the pooled fractions were as follows: fraction 1, 80–150 kDa; fraction 2, 65–80 kDa; fraction 3, 50–65 kDa; fraction 4, 35–50 kDa; fraction 5, 10–35 kDa. All procedures were carried out at 4 °C.

Biochemical assays

The supernatants used for the enzyme assays were obtained in the same way as for the phosphorylation assays. PGM was assayed in the forward direction by modifying the methods of Carreras *et al.* (1982) and Stankiewicz & Haas (1986). The buffer (33 mM-Tris/HCl, pH 7.5, 6 mM-MgCl₂ and 6 mM-KCl) was incubated at 30 °C for 7 min with 0.45 mM-NADH, 2.25 mM-ADP, 20 mM-PCr or equivalent volume of water, 1 mM-3-PG and the homogenate. Then 2.5 units of enolase, 6 units of pyruvate kinase and 16 units of lactate dehydrogenase (which was centrifuged, and the pellet resuspended in assay buffer) were added to the cuvette, and several A_{340} readings were taken as a blank. Finally, the reaction was initiated by addition of 350 μM -2,3-biPG. PGM was assayed in the backward direction by the method of Prehu *et al.* (1988). The buffer (50 mM-triethanolamine, pH 7.5, 10 mM-MgCl₂) was incubated at 30 °C for 7 min with 0.2 mM-NADH, 3 mM-ATP, 0.8 mM-2-PG, equivalent volume of water or 20 mM-PCr, and the homogenate. Then 3.3 units of glyceraldehyde-3-phosphate dehydrogenase and 2 units of phosphoglycerate kinase were added to the cuvette, and several A_{340} readings were taken as a blank. The reactions were initiated by addition of 80 μM -2,3-biPG.

RESULTS

PCr-dependent protein phosphorylation

The effect of PCr on the phosphorylation of cytosolic proteins in the presence of [γ - 32 P]ATP was tested in dialysed cell-free extracts from several rat tissues (Fig. 1, lanes 1 and 2). For comparison, known effectors of protein kinases were added to the extracts in parallel experiments (Fig. 1, lanes 3 and 4). Two proteins of apparent molecular masses 29 and 18 kDa were labelled in a PCr-dependent fashion. There were large tissue-specific differences in the labelling intensity of both proteins. For instance, the 29 kDa protein was intensely labelled in extracts of WGAS muscle, was barely detectable in extracts of soleus muscle, was apparently not affected by the presence of PCr in extracts of heart (Fig. 1) and was not observed in extracts of liver (results not shown). PCr was also observed to decrease the labelling intensity of several proteins which were labelled in control experiments in the presence of [γ - 32 P]ATP alone (e.g. Fig. 1, lane 2). PCr-dependent phosphorylation was restricted to the cytosol; the resuspended 118 000 g pellet showed no PCr-dependent phosphorylation (results not shown). Addition of known protein kinase effectors resulted in the phosphorylation of specific protein substrates; for instance, the α and β subunits of phosphorylase kinase were phosphorylated by cAMP-dependent protein kinase (Fig. 1a, lane 4), and glycogen phosphorylase was phosphorylated in a Ca^{2+} -dependent manner (Fig. 1a, lane 3).

The labelling intensities of the 18 and 29 kDa proteins were unaffected by Ca^{2+} (1.5 mM), cAMP (2 μM)/IBMX (1 mM) (Fig. 1) or Ca^{2+} (1.5 mM)/phosphatidylserine (20 μg) (results not shown) in skeletal muscle. In heart a protein which co-migrated with the 29 kDa protein was intensely labelled in the presence of cAMP. When added in combination with these kinase activators, PCr had little or no effect on the labelling pattern (results not

shown). Labelling patterns in extracts from the muscles of GPA-fed rats were qualitatively similar to those obtained in control animals (results not shown). Further, several effectors of protein phosphatases, namely heparin (8 $\mu\text{g}/\text{ml}$), spermine (0.1 mM), ZnSO_4 (10 μM), MnCl_2 (5 mM), CoCl_2 (5 mM) and heat treatment (reviewed by Ballou & Fisher, 1986), were without effect. Only NaF (50 mM), a phosphatase inhibitor, increased the labelling intensity of the two proteins (results not shown). The concentration of PCr required to produce half-maximal ^{32}P -labelling of the two proteins was of the order of 500 μM (Fig. 2, lanes 1–4). To test whether PCr-dependent phosphorylation was specific to PCr, PGPA, an analogue of PCr, was substituted for PCr in a series of labelling experiments. PGPA added alone did not result in the labelling of either protein (Fig. 2, lanes 5–8), but, when added with PCr, PGPA decreased the labelling intensity of both unknown proteins (Fig. 2, lanes 9–14). The effect was greatest at 20 mM-PGPA and 0.1 mM-PCr (Fig. 2, lane 12), suggesting that PGPA competitively inhibits PCr-dependent phosphorylation.

The presence of impurities in commercial preparations of PCr is known to inhibit phosphofructokinase, pyruvate kinase (Fitch *et al.*, 1979) and adenylate deaminase (EC 3.5.4.6) (Wheeler & Lowenstein, 1979). To test whether PCr-dependent phosphorylation was due to an impurity, commercial PCr was purified by anion-exchange chromatography as in Fitch *et al.* (1979). Purified PCr produced labelled 18 and 29 kDa proteins that could not be distinguished from those labelled by unpurified commercial PCr (results not shown).

To examine the time course of incorporation and stability of the ^{32}P label in the two proteins, phosphorylation reactions were allowed to incubate from 10 to 90 s, and some were chased with unlabelled ATP. Maximum labelling of the 29 kDa protein occurred after 60 s incubation, and the label could not be chased with subsequent addition of unlabelled ATP (Fig. 3). In contrast, labelling of the 18 kDa protein was transient (Fig. 3).

Fig. 1. Autoradiograms of phosphorylated proteins in dialysed cell-free extracts of various rat tissues: (a) WGAS muscle; (b) soleus muscle; (c) heart

Phosphorylation experiments were performed as described in the Materials and methods section. All lanes contained $\sim 20 \mu\text{g}$ of protein and the following additions: no addition (lane 1); 20 mM-PCr (lane 2); 1.5 mM- CaCl_2 (lane 3); 2 μM -cAMP and 1.5 mM-IBMX (lane 4). Arrows at the left of the gel indicate the two proteins phosphorylated in the presence of PCr. White arrowheads point to glycogen phosphorylase (97.4 kDa), phosphorylated in the presence of CaCl_2 (a, lane 3) and to the α and β subunits of phosphorylase kinase (145 kDa and 130 kDa respectively) phosphorylated in the presence of cAMP (a, lane 4). The positions of molecular-mass standards (kDa) are indicated to the right of the gels.

PGM is the unknown 29 kDa protein

The 29 kDa protein co-migrated with a relatively abundant protein detected by staining with Coomassie Blue. PGM, a glycolytic enzyme of 28.5 kDa (Pizer, 1960) which requires phosphorylation for activation (reviewed in Fothergill-Gilmore & Watson, 1989), seemed a possible candidate for the unknown, since commercially available rabbit PGM co-migrated with the unknown band from a rabbit muscle homogenate (results not shown). Several experiments were undertaken to test whether the 29 kDa protein was PGM. First, metabolites involved in the PGM reaction were added to dialysed cell-free extracts to test whether the labelling intensity of the unknown proteins could be altered. Second, known inhibitors of PGM were tested, and finally the nature of the labelled amino acid residue was determined.

In the presence of PCr, high concentrations (10 mM) of 3-PG, 2,3-biPG and 2-PG prevented labelling of the 29 kDa protein (Figs. 4a, 4b and 4c, lane 12); at low concentration (less than 100 μM) the labelling intensity of the 29 kDa protein was unaffected (Fig. 4). The 29 kDa band was not observed on addition of 10 mM-3-PG (Fig. 4a, lane 12), 1 mM-2-PG (Fig. 4b, lane 10) or 100 μM -2,3-biPG (Fig. 4c, lane 8). Interestingly, greater than 100 μM -2,3-biPG also abolished the PCr-independent labelling of several proteins (Fig. 4c, lanes 8, 10, 12).

Sodium vanadate is known to activate the 2,3-biPG phosphatase (EC 3.1.3.13) activity of PGM (Stankiewicz *et al.*, 1987) without affecting the initial phosphorylation event (Carreras *et al.*, 1982), resulting in decreased phosphorylation of PGM and inactivation. Fig. 5(a) clearly shows that the labelling intensity of 29 kDa protein was decreased by the vanadate treatment. Sulphate ions are competitive inhibitors of 2,3-biPG

Fig. 2. Effect of PCr concentration and influence of PGPA on PCr-dependent phosphorylation of the 18 and 29 kDa proteins in WGAS muscle extract

Lanes: 1, no additions; 2, PCr (0.1 mM); 3, PCr (1 mM); 4, PCr (10 mM); 5, PGPA (1 mM); 6, PGPA (10 mM); 7, PGPA (20 mM); 8, PGPA (40 mM); 9, PGPA (10 mM) and PCr (0.1 mM); 10, PGPA (10 mM) and PCr (1 mM); 11, PGPA (10 mM) and PCr (10 mM); 12, PGPA (20 mM) and PCr (0.1 mM); 13, PGPA (20 mM) and PCr (1 mM); 14, PGPA (20 mM) and PCr (10 mM).

Fig. 3. Time course of PCr-dependent phosphorylation and chase with unlabelled ATP

Incubation times were varied to examine the time course of PCr-dependent labelling: 20 mM-PCr was added in all experiments, and incubation times were as follows: 10 s (lane 1); 30 s (lane 2); 60 s (lane 3); 90 s (lane 4). The stability of the phosphorylation was tested by chasing the label with 1 mM-ATP (unlabelled). Proteins were phosphorylated for 30 s (lane 5) and then chased with unlabelled ATP for a further 30 s (lane 6).

binding to PGM (Grisolia & Cleland, 1968), and Fig. 5(b) shows a decreased labelling intensity of the 29 kDa protein in the presence of $(\text{NH}_4)_2\text{SO}_4$. Skeletal-muscle PGM is also inhibited by thiol reagents, which bind to the mono- or bis-phosphorylated-glycerate binding sites (Prehu *et al.*, 1988). The thiol reagent

Fig. 4. Effect of metabolites involved in the PGM reaction on the labelling intensity of the 29 kDa protein in WGAS muscle extracts: (a) 3-PG; (b) 2-PG; (c) 2,3-biPG

Metabolites were added in the following concentrations: zero (lanes 1, 2); 0.1 μM (lanes 3, 4); 1 μM (lanes 5, 6); 100 μM (lanes 7, 8); 1 mM (lanes 9, 10); 10 mM (lanes 11, 12). PCr (20 mM) was added to lanes 2, 4, 6, 8, 10 and 12.

Fig. 5. Effect of PGM inhibitors on the labelling intensity of the 18 and 29 kDa protein in WGAS muscle extracts: (a) sodium vanadate; (b) $(\text{NH}_4)_2\text{SO}_4$; (c) iodoacetamide

No additions were made in lane 1; 20 mM-PCr was added in lanes 2 and 4. Inhibitors were added in lanes 3 and 4 at the following concentrations: 100 μM -sodium vanadate; 0.64 M- $(\text{NH}_4)_2\text{SO}_4$; 2 mM-iodoacetamide.

iodoacetamide produced a decrease in the labelling intensity of the 29 kDa protein (Fig. 5c).

The differential stability of phosphorylated amino acids in acid and base was used to determine the identity of the phosphorylated amino acid in both unknown proteins. Phosphomonoesters ($\text{RCH}_2\text{OPO}_3^{2-}$) are generally acid-stable and base-labile, phosphoramidates ($\text{RCH}_2\text{NHPO}_3^{2-}$) are acid-labile and base-stable, whereas acyl phosphates (RCOOP_3^{2-}) are readily discerned by their lability at both pH extremes (Vogel, 1984). Both treatments produced the expected results for known phosphoserine residues (α and β subunits of phosphorylase kinase,

Fig. 6. Identity of the phosphorylated amino acid residues in the 18 and 29 kDa proteins in WGAS muscle extracts

The gel in (b) was treated with acid and that in (d) with base as described in the Materials and methods section; (a) and (c) are control gels. Proteins were phosphorylated in the presence of the following: no additions (lane 1); 1.5 mM- CaCl_2 (lane 2); 2 μM -cAMP and 1.5 mM-IBMX (lane 3); 20 mM-PCr (lane 4). The 29 and 18 kDa proteins, which are acid-labile and base-stable, are indicated with small arrowheads. Large arrowheads point to glycogen phosphorylase in lane 2 (c, d) and the α and β subunits of phosphorylase kinase in lane 4 (c, d).

Fig. 7. Ability of substrate analogues to dephosphorylate PGM

WGAS muscle supernatants, phosphorylated in the presence of PCr were incubated in the presence of (a) 100 μM -sodium vanadate or (b) 5 mM-2-phosphoglycollate. Initial labelling after 5 min incubation in the presence of 20 mM-PCr is shown in lane 1. Further incubation after addition of 200 μM -ADP (to stop PGM phosphorylation) for 30 s (lane 2), 60 s (lane 3) or 3 min (lane 4). Further incubation in the presence of inhibitors for 30 s (lane 5), 60 s (lane 6) or 3 min (lane 7).

glycogen phosphorylase), which were acid-stable and base-labile (large arrowheads in Figs. 6c and 6d). Both unknowns were acid-labile and base-stable, strongly suggesting that they are both phosphohistidine, since other phosphoramidates (phospho-arginine and phospho-lysine) are not known to be phosphorylated in proteins.

To determine whether the labelled histidine residue was at the PGM active site, the ability of substrate analogues (sodium vanadate and 2-phosphoglycollate) to dephosphorylate the protein was tested. Sodium vanadate mimics 2-PG by binding to PGM at the 2-PG binding site and activating its 2,3-biPG phosphatase activity, resulting in enzyme dephosphorylation and inactivation (Stankiewicz *et al.*, 1987). 2-Phosphoglycollate, a C_2 analogue of 2-PG, is thought to affect PGM in a similar manner (Rose & Dube, 1978). PGM was phosphorylated in the presence of PCr for 5 min, and the reaction was terminated by addition of ADP (final concn. 200 μM). The substrate analogues were added to portions of the reaction mixture and incubated for 30 s–3 min, and the results were compared with a parallel control experiment in which no substrate analogue was added. Addition of either sodium vanadate or 2-phosphoglycollate decreased the labelling intensity of PGM (Figs. 7a and 7b). Addition of 1 mM-2,3-biPG after the initial PCr-dependent phosphorylation also resulted in removal of label from PGM (results not shown).

Fig. 8. Determination of the mechanism of ^{32}P -labelling of PGM in WGAS muscle extracts

Lanes: 1, no addition; 2, PCr (20 mM); 3, phosphoglycerate kinase (18 units); 4, phosphoglycerate kinase (18 units) and PCr (20 mM); 5, PGM (10 units); 6, PGM (10 units) and PCr (20 mM); 7, PGM (10 units) and phosphoglycerate kinase (18 units); 8, PGM (10 units), phosphoglycerate kinase (18 units) and PCr (20 mM).

Mechanism of PGM phosphorylation

PGM when added alone to a dialysed cell-free extract of WGAS muscle did not produce a 29 kDa band (Fig. 8, lane 5), showing that PGM could not phosphorylate itself. However, addition of phosphoglycerate kinase alone or with PGM to muscle extract produced labelled PGM bands without PCr, and enhanced labelling in the presence of PCr (Fig. 8, lanes 3 and 4). The largest enhancement of PCr-dependent labelling was observed when both phosphoglycerate kinase and PGM were added (Fig. 8, lane 8). Similarly, labelled PGM could be produced in a model system consisting of [γ - ^{32}P]ATP, phosphoglycerate kinase, PGM and 3-PG, without tissue extract or PCr (results not shown). PCr-dependent phosphorylation in cytosolic muscle extracts required a muscle-specific factor which was not removed by dialysis. Creatine kinase is the only known protein for which PCr is a substrate. To test whether creatine kinase was essential for PCr-dependent phosphorylation of PGM, undialysed WGAS muscle supernatant was fractionated on a Sephadex G-100 column and the various fractions were tested for their ability to support PCr-dependent phosphorylation. Fig. 9 shows a very small amount of PCr-independent labelling of PGM in fractions 1–3. PCr-dependent PGM labelling was observed in fractions 1 and 2 only. Measurements of PGM enzyme activity showed that most of the activity was eluted in these fractions; SDS/PAGE analysis showed that PGM co-eluted with creatine kinase (results not shown). In the presence of exogenous PGM, labelled PGM was also observed in fractions 3 and 4 on addition of PCr, but not in fraction 5 (Fig. 9). Creatine kinase was undetectable in this fraction.

Effect of PCr on PGM activity

The effect of PCr on the enzyme activity of PGM was measured in the forward and reverse direction in dialysed supernatants from WGAS muscle. PCr produced an artifactual inhibition of PGM activity in the forward direction (317 ± 48 and $0 \mu\text{mol}$ of substrate transformed/min per g wet wt. for control and plus PCr respectively), owing to a decrease in the ADP pool used in

Fig. 9. Ability of size-fractionated supernatant from rat WGAS muscle to support phosphorylation of PGM and the 18 kDa protein

The approximate molecular-mass range covered by the pooled fractions was as follows: fraction 1, 80–150 kDa; fraction 2, 65–80 kDa; fraction 3, 50–65 kDa; fraction 4, 35–50 kDa; fraction 5, 10–35 kDa. Fractions 1–5, no additions (lanes 1–5). Fractions 1–5 plus 20 mM-PCr (lanes 6–10). Fractions 1–5 plus 20 mM-PCr and PGM (10 units) (lanes 11–15).

the coupled enzyme system, but did not affect the activity of the enzyme assayed in the reverse direction (81 ± 37 and 79 ± 23 μmol of substrate transformed/min per g wet wt. for control and plus PCr respectively).

Phosphorylation of the 18 kDa protein

The 18 kDa protein was observed to be phosphorylated on a histidine residue (Fig. 6). Phosphorylation of the 18 kDa protein was unaffected by high concentrations of 2,3-biPG, but did not occur at high concentrations (10 mM) of 3-PG and 2-PG (Figs. 4a and 4b, lane 12). Inhibitors of PGM had no effect on labelling of the 18 kDa protein (Fig. 5). In contrast with PGM, labelling of the 18 kDa protein was transient (Fig. 3). The phosphorylation state of the 18 kDa protein was dependent on PGM in fractionated WGAS supernatant. Fig. 9 shows a small amount of PCr-independent phosphorylation of the 18 kDa protein in fractions 1–4, which was not changed on addition of PCr. However, addition of PGM and PCr enhanced labelling of the 18 kDa protein, especially when PGM labelling was weak or absent (Fig. 9, lanes 13–15).

DISCUSSION

Two cytosolic proteins of apparent molecular mass 18 and 29 kDa were shown to be phosphorylated in the presence of [γ - ^{32}P]ATP in a PCr-dependent fashion in cell-free extracts of skeletal muscle. The phosphorylation state of the two proteins was not affected by activators of protein kinases A or C or by effectors of protein phosphatase, except NaF (a phosphatase inhibitor), suggesting that PCr-dependent phosphorylation occurred via a unique kinase/phosphatase system or that the proteins were phosphoenzyme intermediates. PGM, a homodimeric enzyme in the glycolytic pathway, of native molecular mass 57 kDa (Pizer, 1960), which is known to require phosphorylation on an active-site histidine residue for activity, was shown to be the 29 kDa unknown.

PGM catalyses the interconversion of 3-PG and 2-PG and requires 2,3-biPG for activation. 2,3-biPG potentiates catalysis by donating one of its phosphate groups to a histidine residue present at the enzyme active site, transiently forming a phosphorylated enzyme. A monophosphoglycerate molecule (resulting from the exchange of a phospho group between 2,3-biPG and PGM) then leaves the active site. Catalysis begins when either 3-PG or 2-PG binds at the active site. Substrate binding at the active site results in 2,3-biPG re-formation and reorientation. This allows the enzyme to be phosphorylated by the phosphate group from the incoming substrate, ultimately resulting in phosphate-group transfer on the glycerate molecule

(reviewed in Fothergill-Gilmore & Watson, 1989). Phosphorylation of PGM by 2,3-biPG is an integral part of the enzyme activation; there are no known allosteric phosphorylation events which influence PGM activity. The concentration of 2,3-biPG in the cell is controlled by 2,3-biPG mutase and 2,3-biPG phosphatase, which catalyse the synthesis and breakdown of 2,3-biPG respectively. PGM and these two enzymes possess, to a limited extent, activities of the other two enzymes (Rose & Dube, 1976).

Several lines of evidence led to the demonstration of PGM as the 29 kDa protein. (1) Commercial PGM co-migrated with the unknown protein (2) The labelling intensity of the 29 kDa protein was altered predictably in response to treatment with sodium vanadate, sulphate ion, iodoacetamide and 2-phosphoglycollate. The effect of NaF treatment (increased labelling intensity of the 29 kDa protein) can be attributed to the inhibition of 2,3-biPG phosphatase by NaF (Narita *et al.*, 1979). (3) The protein was phosphorylated at the active-site histidine residue. (4) The labelling intensity of the 29 kDa protein could be altered predictably by metabolites involved in the PGM reaction. 3-PG, 2,3-biPG and 2-PG all decreased the intensity of the 29 kDa band, likely owing to dilution of the specific radioactivity of labelled 2,3-biPG.

Transfer of [^{32}P]phosphate from ATP to 2,3-biPG and then to PGM can occur via phosphoglycerate kinase and 2,3-biPG mutase (reactions 2 and 7, Fig. 10). This mechanism requires that a residual amount of 3-PG be present in the supernatant after dialysis. The validity of this mechanism is suggested by the following observations. (1) Purified PGM was not phosphorylated when added alone in a phosphorylation assay. (2) The end product of these reactions is the metabolite known to phosphorylate PGM at the active-site histidine residue. (3) Addition of 2-PG, 3-PG or 2,3-biPG prevented PGM labelling, and 2,3-biPG was capable of removing the phosphate label from PGM after PCr treatment. (4) Addition of phosphoglycerate

Fig. 10. Diagram of the reactions involved in or pertinent to protein phosphorylations observed in this study

(1) Glyceraldehyde-3-phosphate dehydrogenase; (2) phosphoglycerate kinase; (3) PGM; (4) enolase; (5) pyruvate kinase; (6) lactate dehydrogenase; (7) 2,3-biPG mutase; (8) creatine kinase.

kinase or phosphoglycerate kinase and PGM to muscle extract in a phosphorylation experiment resulted in labelled PGM in the absence of PCr and enhanced labelling in its presence. (5) PGM phosphorylation could be reproduced in a model system without added muscle extract. What is the effect of PCr on this mechanism? PCr did not affect the system at the level of phosphoglycerate kinase, as the enzyme activity of phosphoglycerate kinase was not altered by PCr (results not shown). Rather, PCr, in the presence of creatine kinase, shifted the equilibrium of the phosphoglycerate kinase reaction towards synthesis of 1,3-biPG, by removing ADP, ultimately resulting in an increase in labelled 2,3-biPG. The involvement of creatine kinase is supported by the results obtained in size-fractionated muscle supernatant. Only those fractions containing both PGM and creatine kinase supported PCr-dependent PGM labelling.

This mechanism can also account for the PCr-dependent decrease in labelling intensity observed in several unidentified proteins. Creatine kinase, in the presence of PCr and ADP, catalyses the transfer of a phosphate group from PCr to ADP to form ATP and creatine. The ATP formed from this reaction would not be ^{32}P -labelled, and would therefore decrease the specific radioactivity of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ pool. This would result in decreased labelling intensity of the proteins directly labelled by ATP. However, the fact that most PCr-independent protein phosphorylation was eliminated on addition of greater than $100\text{ }\mu\text{M}$ -2,3-biPG suggests that these proteins are not labelled directly by ATP, but rather by metabolites derived from 2,3-biPG. The scheme also predicts that PGPA would not modulate the phosphorylation of PGM protein, since PGPA is a very poor substrate for creatine kinase (Chevli & Fitch, 1979) and would therefore leave the ADP pool unperturbed.

PCr-dependent phosphorylation was observed to be tissue-specific for both proteins. PGM was intensely labelled in extracts of WGAS muscle, barely detectable in extracts of soleus and not affected in extracts of heart. This difference in labelling pattern probably reflects differences in the activities of phosphoglycerate kinase and 2,3-biPG mutase, which are necessary to synthesize 2,3-biPG, and in the amount of PGM available for phosphorylation. Phosphoglycerate kinase activity in skeletal muscle is about 2-fold higher than in the heart (Newsholme & Start, 1976) and the activity of 2,3-biPG mutase is about 50-fold higher (Tauler *et al.*, 1987a); thus one would predict greater availability of labelled 2,3-biPG in skeletal muscle. PCr was observed to have no effect on the phosphorylation state of PGM in heart homogenates, but nevertheless PGM appeared to be phosphorylated. Although the presence of another phosphorylatable protein of 29 kDa does not permit unequivocal interpretation of this result, it is possible that increased 2,3-biPG levels from blood contamination in the heart (dialysis would remove endogenous 2,3-biPG, but not the enzymes responsible for its biosynthesis) were high enough to inhibit 2,3-biPG mutase (Chiba & Sasaki, 1978). The weak labelling of PGM in the soleus muscle compared with gastrocnemius muscle extracts is likely due, at least in part, to the 6-fold greater concentration of PGM in fast-twitch glycolytic as compared with slow-twitch oxidative muscles (Andres *et al.*, 1989).

The identity of the 18 kDa protein remains unknown; however, a number of pieces of evidence suggest that it is a phosphoenzyme intermediate labelled by 1,3-biPG. (1) The 18 kDa protein was phosphorylated at a histidine residue. (2) In the presence of PCr, 10 mM-3-PG or -2-PG resulted in the disappearance of the 18 kDa band; however, a high concentration of 2,3-biPG neither affected the labelling intensity nor chased the label, showing that a metabolite derived from 2- or 3-PG, but not 2,3-biPG, was responsible for labelling. (3) The transient nature of the phosphorylation suggests that the metabolite is an intermediate

between ATP and 2,3-biPG. (4) The labelling intensity of the 18 kDa protein was dependent on the relative labelling intensity of PGM and independent of creatine kinase in size-fractionated muscle extracts. Labelling was increased in the presence of PCr when PGM labelling was undetectable, conditions that would produce an increase in labelled 1,3-biPG, possibly owing to a low activity of 2,3-biPG mutase. It is thus very likely that the 18 kDa protein is a phosphoenzyme intermediate labelled by $[\text{1-}^{32}\text{P}]\text{1,3-biPG}$. This hypothesis is further supported by results obtained in the heart supernatant in which labelling of the 18 kDa protein, but not PGM, was markedly increased on addition of PCr.

There have been a number of previous reports of cytosolic proteins whose phosphorylation state can be modulated by metabolites in the glycolytic pathway such as phosphoenolpyruvate, 2-PG and 3-PG (Khandelwal *et al.*, 1983; Dworkin & Dworkin-Rastl, 1987; Ueda & Plagens, 1987; Pek *et al.*, 1990; Morino *et al.*, 1991). Ueda and co-workers studied the 3-PG-dependent phosphorylation of several unidentified proteins in a variety of mammalian tissues (Ueda & Plagens, 1987; Pek *et al.*, 1990), focusing on the 3-PG-dependent phosphorylation of 72 and 155 kDa proteins in the bovine brain. Although they attributed these 3-PG-dependent phosphorylations to the action of novel kinases (Ueda & Plagens, 1987), many (if not all) of these proteins are likely to be enzyme intermediates, phosphorylated by bisphosphorylated metabolites which become labelled in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, namely 1,3-biPG, 2,3-biPG, glucose 1,6-bisphosphate (G-1,6-biP) or fructose 2,6-bisphosphate (F-2,6-biP), in a manner similar to that observed in this study. Some of the 3-PG-dependent phosphorylated proteins (Ueda & Plagens, 1987; Pek *et al.*, 1990) can be identified on the basis of their monomeric molecular mass and their known affinities for metabolites which could have become radioactively labelled from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the cell-free cytosolic extracts. For example, a 65 kDa protein which was phosphorylated in the presence of 2- or 3-PG in pancreatic islets (Pek *et al.*, 1990) is very likely phosphoglucomutase (EC 5.4.2.2). Dworkin & Dworkin-Rastl (1987) observed that addition of $[\text{3-}^{32}\text{P}]\text{phosphoenolpyruvate}$ to cell-free extracts resulted in the phosphorylation of a 29 kDa protein and subsequently a 65 kDa protein, which they identified as PGM and phosphoglucomutase respectively. In the study by Pek *et al.* (1990), phosphoglucomutase labelling could have occurred via $[\text{1-}^{32}\text{P}]\text{1,3-biPG}$ formed in the phosphoglycerate kinase reaction, which subsequently labelled G-1,6-biP through reactions in the gluconeogenic pathway. Indeed, Dworkin & Dworkin-Rastl (1987) have suggested that the relative labelling intensity of phosphoglucomutase in such an experiment should reflect the relative gluconeogenic capacity of the tissue.

Ueda and co-workers (Morino *et al.*, 1991) very recently showed that the 72 kDa protein whose labelling was 3-PG-dependent is G-1,6-biP synthase, directly labelled by $[\text{1-}^{32}\text{P}]\text{1,3-biPG}$. They reported that the 155 kDa protein was not phosphorylated by 1,3-biPG, but did not address the possibility that either G-1,6-biP or F-2,6-biP could phosphorylate the 155 kDa unknown. F-2,6-biPase (EC 3.1.3.46), G-1,6-biP synthase and phosphoglucomutase appear to be the only known proteins, other than the enzymes involved in 2,3-biPG metabolism, that can be phosphorylated by 1,3-biPG. 1,3-biPG phosphorylates F-2,6-biPase bisphosphatase at an active-site histidine residue, and is capable of triggering the dephosphorylation of 1,3-biPG to 3-PG and P_i (Tauler *et al.*, 1987b). This activity has been attributed to amino acid homology in the active sites of fructose-2,6-phosphatase, PGM and 2,3-biPG mutase (Tauler *et al.*, 1987b). Bartrons *et al.* (1985) reported that 1,3-biPG is able to phosphorylate phosphoglucomutase and may activate the enzymic reaction in the absence of G-1,6-biP. It is unlikely that the 18 kDa protein is any of these, as the

monomeric molecular mass of F-2,6-biPase is 55 kDa (Pilkis *et al.*, 1983), that of G-1,6-biP synthase is 72 kDa (Morino *et al.*, 1991) and that of phosphoglucomutase is 66 kDa (Dworkin & Dworkin-Rastl, 1987). Our results suggest that there is at least one other protein which can be phosphorylated by 1,3-biPG.

PGM, phosphoglucomutase, F-2,6-biPase, G-1,6-biP synthase, 2,3-biPG mutase and 2,3-biPG phosphatase catalyse reactions in which bisphosphorylated metabolites play an essential role in the reaction mechanism. All members of this group of enzymes possess a bisphosphorylated-metabolite-binding domain, which is likely to have evolved from a common ancestor, and which is capable of binding several bisphosphorylated metabolites with different affinities. For example, phosphoglucomutase can bind F-1,6-biP, F-2,6-biP, 1,3-biPG, 2,3-biPG and G-1,6-biP (Bartrons *et al.*, 1985). Although there is significant amino acid sequence homology between some members of this group of enzymes (e.g. F-2,6-biPase and PGM and 2,3-biPG mutase; Tauler *et al.*, 1987b), others, such as PGM and phosphoglucomutase, are clearly not related. A possible homology of the 1,3-biPG binding domains has, however, been suggested between glycerate 2,3-biPG synthase/phosphatase and G-1,6-biPG synthase (Rose, 1986). This group of enzymes might include those that do not appear to be part of the glycolytic pathway, such as the 18 kDa protein observed in this study.

Although we have shown that PCr can modulate the phosphorylation state of PGM and an 18 kDa protein in dialysed cell-free extracts of skeletal muscle, regulation of PGM *in vivo* by PCr is only likely to be important indirectly through stabilization of ADP levels by the buffering action of PCr, since PCr has no direct effect on PGM activity. This study could not ascertain any direct role for PCr in the metabolic alterations observed in PCr-depleted rat muscles. The molecular mechanisms by which skeletal muscles adapt to the loss of a cellular energy buffer remain unknown.

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