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Phosphoglycerates and Protein Phosphorylation: Identification of a Protein Substrate as Glucose-1,6-Bisphosphate Synthetase

*Hideo Morino, *Carolyn Fischer-Bovenkerk, *Phillip E. Kish, and *†Tetsufumi Ueda

**Mental Health Research Institute and †Departments of Pharmacology and Psychiatry, University of Michigan, Ann Arbor, Michigan, U.S.A.*

Abstract: We have previously reported the occurrence of two endogenous protein phosphorylation systems in mammalian brain that are enhanced in the presence of 3-phosphoglycerate (3PG) and ATP. We present here a study of one of these systems, the phosphorylation of the 72-kDa protein (3PG-PP_{72}). This system was separated into the substrate, 3PG-PP_{72}, and a kinase by ammonium sulfate fractionation, hydroxyapatite chromatography, and hydrophobic interaction HPLC. The substrate protein was shown to be directly phosphorylated with [1-^{32}P]1,3-bisphosphoglycerate ([1-^{32}P]1,3BPG) with an apparent K_m of 1.1 n*M*. Nonradioactive 1,3BPG inhibited ^{32}P incorporation in the presence of [γ-^{32}P]ATP and 3PG. Phosphopeptide mapping and phosphoamino acid analyses indicated that the site of phosphorylation of 3PG-PP_{72} observed in the presence of 3PG and ATP is a serine residue identical to that observed with [1-^{32}P]1,3BPG. Moreover, [^{32}P]phosphate incorporated into 3PG-PP_{72} in the presence of 3PG and ATP was removed by subsequent incubation with glucose-1-phosphate or glucose-6-phosphate. Finally, 3PG-PP_{72} showed chromatographic behaviors identical to those of glucose-1,6-bisphosphate ($G1,6P_2$) synthetase. Based upon these observations, we conclude that 3PG-PP_{72} is $G1,6P_2$ synthetase and that it is phosphorylated directly by 1,3BPG, which is formed from 3PG and ATP by 3PG kinase present in a crude 3PG-PP_{72} preparation. **Key Words:** Protein phosphorylation—3-Phosphoglycerate—Phosphoprotein—Glucose-1,6-bisphosphate synthetase—1,3-Bisphosphoglycerate—Brain. **Morino H. et al.** Phosphoglycerates and protein phosphorylation: Identification of a protein substrate as glucose-1,6-bisphosphate synthetase. *J. Neurochem.* **56,** 1049–1057 (1991).

Glucose metabolism is of vital importance for brain function. Although glucose is the major substrate for energy production in the brain under normal conditions (Siesjö, 1978), it is now apparent that ATP alone is not sufficient to maintain synaptic function (Dirks et al., 1980; Winkler, 1981; Cox and Bachelard, 1982; Ghajar et al., 1982). This information raised the possibility that glycolysis or a glycolytic intermediate, in addition to ATP, might play a role in synaptic transmission in normal physiological states. In view of these considerations, together with the growing evidence for the role of protein phosphorylation in the function of the nervous system (Nestler and Greengard, 1984), a search was made for a potential link between glycolysis and protein phosphorylation (Ueda and Plagens, 1987). It was found that 3-phosphoglycerate (3PG), one of the glycolytic intermediates, stimulates the phosphorylation of two specific proteins in the brain with molecular weights of 72,000 and 155,000, referred to as 3PG-PP_{72} and 3PG-PP_{155}. Toward an understanding of the nature of one of the phosphoproteins, 3PG-PP_{72}, we have carried out its purification in this study.

During the course of purifying 3PG-PP_{72}, we noted that its molecular weight in sodium dodecyl sulfate (SDS) was similar to that of glucose-1,6-bisphosphate ($G1,6P_2$) synthetase, which is phosphorylated by 1,3-bisphosphoglycerate (1,3BPG) (Rose et al., 1975). $G1,6P_2$ synthetase was found to be present at high concentrations in the brain (Rose et al., 1975, 1977), as is the case for 3PG-PP_{72} (Ueda and Plagens, 1987). In addition, the reported ammonium sulfate fractionation and hydroxyapatite chromatographic behavior of

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Address correspondence and reprint requests to Dr. T. Ueda at Mental Health Research Institute, University of Michigan, 205 Washtenaw Pl., Ann Arbor, MI 48109, U.S.A.

The present address of Dr. H. Morino is Department of Neuropsychiatry, Ehime University School of Medicine, Shizukawa, Shigenobu-cho, Onsen-gan, Ehime 791-02, Japan.

Abbreviations used: 1,3BPG, 1,3-bisphosphoglycerate; $G1,6P_2$, glucose-1,6-bisphosphate; PAGE, polyacrylamide gel electrophoresis; 3PG, 3-phosphoglycerate; 3PG-PP_{72}, 72-kDa protein phosphorylated in presence of 3PG; 3PG-PP_{155}, 155-kDa protein phosphorylated in presence of 3PG; SDS, sodium dodecyl sulfate.

$G1,6P_2$ synthetase (Wong and Rose, 1976) appeared similar to that of $3PG\text{-}PP_{72}$. These observations raised the possibility that $3PG\text{-}PP_{72}$ might be phosphorylated directly by 1,3BPG, which could have been formed from 3PG and ATP catalyzed by an endogenous source of enzyme, which could be 3PG kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3), and that $3PG\text{-}PP_{72}$ might be $G1,6P_2$ synthetase. Hence, we set out to determine the identity of $3PG\text{-}PP_{72}$ as $G1,6P_2$ synthetase. The potential role of $G1,6P_2$ synthetase in synaptic function is discussed. We have also determined whether $3PG\text{-}PP_{155}$ is phosphorylated directly by 1,3BPG.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (7,000 Ci/mmol), [^{32}P]P_i (285 Ci/mg), and gel electrophoresis reagents were obtained from ICN Biomedicals. Spherogel TSK Phenyl 5PW and Ultrasil SAX columns were purchased from Beckman Instruments. Biogel HTP (hydroxyapatite), Econo-Pak 10DG, and molecular weight standards were from Bio-Rad Laboratories. Glycolytic intermediates and all other chemicals were obtained from Sigma.

Protein phosphorylation assay

The phosphorylation assay was carried out as described by Ueda and Plagens (1987). The standard incubation mixture contained, in a final volume of 100 μl, 5 μmol of Tris-HCl (pH 7.4), 1 μmol of $MgSO_4$, 0.5 μmol 3PG, 2 nmol of [γ-^{32}P]ATP (16.5 Ci/mmol), and appropriate amounts of synaptosomal cytosol or other samples after various steps of purification. When fractions were assayed for the presence of substrate, a small amount of exogenous microsomal kinase (preparation described below) was added. When $3PG\text{-}PP_{72}$ was phosphorylated by [1-^{32}P]1,3BPG, the incubation mixture contained, in a final volume of 100 μl, 5 μmol of Tris-HCl (pH 7.4), 1 μmol of $MgSO_4$, 1 nmol of [1-^{32}P]1,3BPG (10.6 Ci/mol), and an appropriate amount of the fraction containing $3PG\text{-}PP_{72}$. Slab SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography were carried out as described by Ueda and Greengard (1977), and the amounts of [^{32}P]phosphate incorporated into $3PG\text{-}PP_{72}$ were estimated by the densitometric method as described in Ueda et al. (1973) or determined directly by excising and measuring the radioactivity of the portion of the gel corresponding to $3PG\text{-}PP_{72}$.

Preparation of microsomal source of kinase

A kinase that phosphorylates specific proteins in the presence of 3PG and ATP has been found in a particulate fraction rich in microsomes from rat brain (Ueda and Plagens, 1987). Fresh rat cerebrum was homogenized in 10 vols of 0.32 *M* sucrose and subjected to differential centrifugation to obtain the microsomal pellet. The pellet, obtained after centrifuging at 150,000 g_{max} for 60 min, was resuspended in 10 vols of 0.32 *M* sucrose. The kinase was extracted from the microsome suspension with 0.2% Triton X-100 on ice for 30 min. The mixture was then centrifuged at 150,000 g_{max} for 60 min and the clear supernatant was stored at −20°C and used as an exogenous source of 3PG-dependent kinase.

Preparation of synaptosomal cytosol

Fresh bovine brains were obtained from a local slaughterhouse, transported on ice, and processed immediately. The synaptosmal cytosol fraction was prepared as described previously (Ueda et al., 1979). Bovine cerebral cortex was homogenized in an isotonic 0.32 *M* sucrose solution and the synaptosomal (pinched-off nerve endings) fraction was prepared by successive differential and sucrose density gradient centrifugations. The synaptosome-rich fraction was lysed at pH 8.1 (6 m*M* Tris-maleate) and centrifuged at 36,000 g_{max} for 20 min. The resulting supernatant was centrifuged at 150,000 g_{max} for 60 min to pellet synaptic vesicles and small plasma membrane fragments. The supernatant (synaptosomal cytosol fraction) was frozen at −20°C.

Separation of 1,3BPG from ATP and P_i

A Beckman Ultrasil SAX column (250 × 4.6 mm) HPLC system was used for the separation of 1,3BPG, ATP, and P_i and a wavelength of 214 nm was selected to monitor the eluate from the column. The column was equilibrated with 0.4 *M* potassium phosphate buffer (pH 3.2) for >1 h at a flow rate of 1.0 ml/min. Samples were applied in 100 μl and eluted with the same buffer at a flow rate of 1.0 ml/min and eluates were collected in 1-ml fractions. 1,3BPG showed a retention time of 12.6 min. Retention times for the other related compounds were 3.5 min for P_i and glyceraldehyde-3-phosphate, 3.7 min for pyruvate, 4.9 min for 3PG, 21.9 min for 2,3-bisphosphoglycerate, and 22.5 min for ATP. The 1,3BPG peak fraction was identified using both radioactive and nonradioactive 1,3BPG, which had each been synthesized enzymatically. The nonradioactive 1,3BPG, which was dissolved in 10 m*M* glycylglycine buffer (pH 7.5) containing 0.2 *M* NaCl, showed one major peak (retention time, 12.6 min) and two minor peaks (retention times, 3.5 and 4.9 min) of absorbance at 214 nm, in addition to the peak corresponding to glycylglycine (retention time, 3.2 min). The radioactive sample exhibited a major peak of radioactivity with retention time of 12.6 min. When this radioactive substance was treated with acid-molybdate in the presence of isobutanol/benzene, the radioactivity was recovered in the organic phase, which suggested the release of $^{32}P_i$ under acid conditions (Wong and Rose, 1976). These observations indicated that the substance in the major peak fraction was 1,3BPG, and therefore it was used as 1,3BPG.

Synthesis of [1-^{32}P]1,3BPG

[1-^{32}P]1,3BPG and nonradioactive 1,3BPG were synthesized according to an enzymatic method of Rose (1968) except that the enzymes were removed at the end of synthesis by the use of an Amicon Centriprep-10 concentrator. Radioactivity that amounted to 90% of the original $^{32}P_i$ radioactivity was recovered. The specific activity and purity of [1-^{32}P]1,3BPG were determined using an HPLC system. Sixty-eight percent of the total radioactivity of the [1-^{32}P]1,3BPG preparation was associated with [1-^{32}P]1,3BPG, while 8.0% was with P_i. The specific activity was 10.6 Ci/mmol and the yield was 61.2%. The specific activity was lower than that of P_i added to the incubation mixture (40.3 Ci/mmol). This was due to the decomposition of some of the glyceraldehyde-3-phosphate to P_i and glyceraldehyde; thus, the resulting specific activity of P_i was decreased in the incubation mixture. It was calculated that the decomposition of 1.33% of glyceraldehyde-3-phosphate per 25 min at 25°C (data supplied from Sigma) could lead to the decreased specific activity of P_i in the incubation mixture. Nonradioactive 1,3BPG was determined according to Negelein et al. (1957) and used as the standard for the determination of the radioactive 1,3BPG by HPLC. The yield of the nonradioactive preparation was 32.8% and it contained ~12% 3PG and P_i.

Assay for G1,6P$_2$ synthetase and 3PG kinase

G1,6P$_2$ synthetase was assayed according to Wong and Rose (1976). Activity of 3PG kinase was measured according to Scopes (1975).

Peptide mapping and phosphoamino acid analysis of 3PG-PP$_{72}$

The 3PG-PP$_{72}$ fraction from hydroxyapatite chromatography or a mixture of the 3PG-PP$_{72}$ fraction and a kinase fraction from HPLC on the Phenyl 5PW column was incubated with either [γ-^{32}P]ATP or [1-^{32}P]1,3BPG. Phosphorylated samples (250 μl) were subjected to SDS-PAGE. Limited proteolytic digestion and two-dimensional mapping were performed as described by Stratford et al. (1984) and Huttner and Greengard (1979). Tryptic and chymotryptic digests were also subjected to phosphoamino acid analysis according to Stratford et al. (1984).

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard. The amount of 3PG-PP$_{72}$ in the 3PG-PP$_{72}$ fraction from the two chromatographies was determined by densitometric tracing of the Coomassie Blue-stained protein band that corresponded to the band of 3PG-PP$_{72}$ on autoradiogram. Bovine serum albumin was used as a standard.

RESULTS

Separation of 3PG-PP$_{72}$ and endogenous kinase

Preparation of fraction B. The synaptosomal cytosol isolated from six bovine brains was thawed and then fractionated with ammonium sulfate at 0–35% (fraction A), 35–60% (fraction B), and 60–100% (fraction C) saturation. Each fraction was dissolved in a small amount of 10 m*M* Tris-HCl (pH 7.4). After this procedure and all subsequent purification steps, fractions were assayed for enzyme activity as described above. Fraction B contained the majority of 3PG-PP$_{72}$ and fraction A contained 3PG-PP$_{155}$. Each fraction showed endogenous protein phosphorylation in the presence of 3PG and ATP that was not increased by addition of the exogenous source of kinase.

Hydroxyapatite column chromatography. Fraction B was dialyzed against 4 L of 5 m*M* sodium phosphate buffer (pH 7.4) and applied to a hydroxyapatite column (1.5 × 30 cm) equilibrated with the same buffer. After the column was washed with 150 ml of 5 m*M* sodium phosphate buffer (pH 7.4), the elution was performed with a 600-ml linear gradient made of 300 ml each of 5 m*M* and 150 m*M* sodium-potassium phosphate buffer (pH 7.4) at a flow rate of 100 ml/h. Fractions of 10 ml each were collected and aliquots (60 μl) assayed for kinase and substrate. 3PG-PP$_{72}$ appeared as a single peak (Fig. 1, fractions 35–38); these fractions still showed significant endogenous protein phosphorylation with 3PG and ATP, although it was increased by the addition of the exogenous source of kinase.

Spherogel TSK Phenyl 5PW HPLC. The 3PG-PP$_{72}$-containing fractions were pooled and solid ammonium sulfate was added slowly to the fractions to give 80% saturation. After 1 h of stirring at 4°C, the precipitate was collected by centrifugation at 120,000 g_{max} for 30 min. The resulting pellet was dissolved in 0.1 *M* sodium phosphate (pH 7.0) and applied to an Econo-Pak 10DG column that had been equilibrated with 0.1 *M* sodium phosphate (pH 7.0) containing 1 *M* ammonium sulfate.

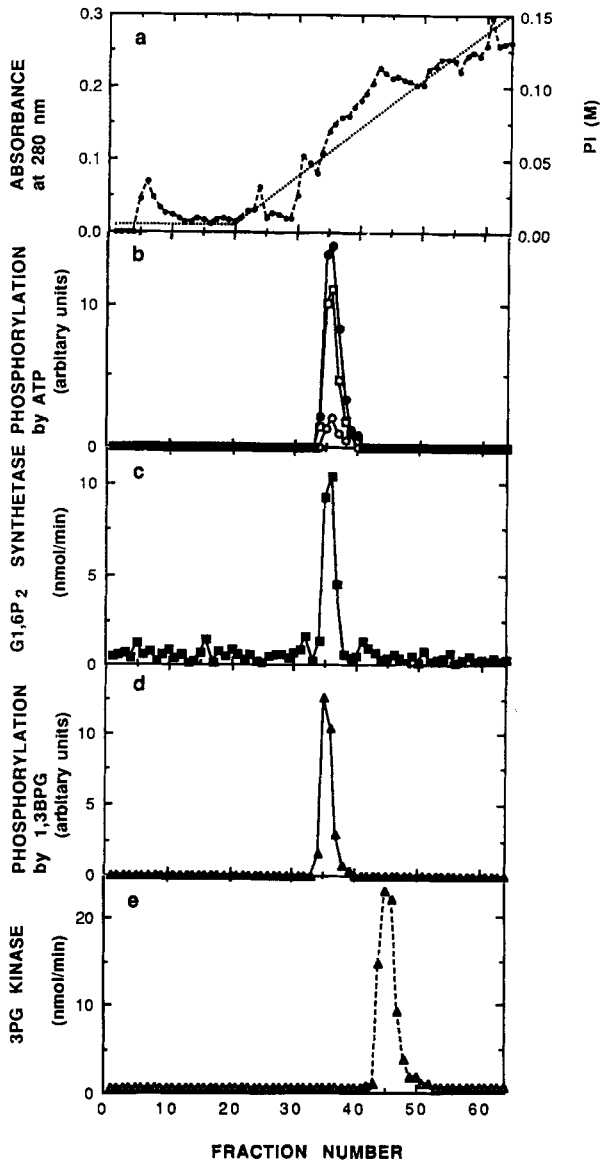

FIG. 1. Hydroxyapatite chromatography of 3PG-PP$_{72}$ and G1,6P$_2$ synthetase. **a:** Profiles of absorbance at 280 nm and the gradient of phosphate concentration. **b:** Phosphorylation of 3PG-PP$_{72}$ by ATP. An aliquot (25 μl) of each fraction was incubated with [γ-^{32}P]ATP in the absence (○) and presence (□) of 3PG or in the presence of 3PG plus exogenous 3PG-dependent kinase (4 μl; ●) for 5 min. Samples were subjected to SDS-PAGE and autoradiography. ^{32}P incorporation into 3PG-PP$_{72}$ was estimated by the densitometric method. **c:** Activity of G1,6P$_2$ synthetase. An aliquot (50 μl) was used to measure the activity of G1,6P$_2$ synthetase. **d:** Phosphorylation of 3PG-PP$_{72}$ by [1-^{32}P]1,3BPG. An aliquot (50 μl) was incubated with [1-^{32}P]1,3BPG (10 μ*M*) for 5 min and subjected to SDS-PAGE and autoradiography. ^{32}P incorporation into 3PG-PP$_{72}$ was estimated by the densitometric method. **e:** Activity of 3PG kinase. An aliquot (400 μl) was used.

The protein was eluted according to the instruction manual. The eluate was applied to a Spherogel TSK Phenyl 5PW column (0.75 × 7.5 cm) that had been attached to a Beckman HPLC system and equilibrated with the buffer containing 1 *M* ammonium sulfate. The column was washed with 24 ml of the same salt buffer. Then, 3PG-dependent kinase and its substrate protein were eluted from the column by decreasing the concentration of ammonium sulfate linearly from 1.0 to 0 *M* in 60 ml of the same buffer (30 ml of each) at a flow rate of 1.0 ml/min. Fractions of 3 ml each were collected. After dialysis against 5 m*M* Tris-HCl buffer (pH 7.4), aliquots of these fractions were assayed for the presence of substrate, using the exogenous source of kinase. 3PG-PP_{72} appeared as a single peak (fractions 23 and 24), as shown in Fig. 2. These fractions showed only low levels of endogenous phosphorylation with 3PG and ATP, indicating a minimal contamination of kinase. When each fraction was assayed for the 3PG-dependent kinase activity, using the 3PG-PP_{72} fractions largely free of kinase as a substrate, the most prominent kinase activity was observed in fractions 28 and 29, although multiple peaks of kinase activity were detected.

As shown in Fig. 3, the addition of fraction 28 to the substrate fraction (fraction 24) increased markedly the protein phosphorylation in the presence of 3PG and ATP of 3PG-PP_{72}, although each alone showed little or no detectable phosphorylation. These results support the notion that the substrate protein 3PG-PP_{72} that utilizes ATP and 3PG and a kinase exist as separate entities.

Identification of 3PG-PP_{72} as G1,6P_2 synthetase

Chromatographic profile. Most of the G1,6P_2 synthetase activity was found in fraction B after the ammonium sulfate fractionation, and this fraction also contained most of the 3PG-PP_{72}. In addition, both G1,6P_2 synthetase and 3PG-PP_{72} exhibited essentially identical elution profiles on hydroxyapatite and hydrophobic interaction (Phenyl 5PW) chromatography (Figs. 1 and 2). Moreover, 3PG-PP_{72} was labeled by [1-^{32}P]1,3BPG (see Figs. 1, 2, 5, 7, and 9) as was G1,6P_2 synthetase. These results suggested that 3PG-PP_{72} is G1,6P_2 synthetase.

Removal of ^{32}P from 3PG-PP_{72} by glucose-1-phosphate or glucose-6-phosphate. Since it has been shown that the phosphoryl group can be removed from the phosphoryl intermediate enzyme of G1,6P_2 synthetase by incubation with glucose-1-phosphate or glucose-6-phosphate as a phosphoryl group acceptor (Rose et al., 1977), we tested a variety of glycolytic intermediates and related compounds for the ability to dephosphorylate 3PG-PP_{72}. After the fraction from hydroxyapatite chromatography was incubated with 3PG and [γ-^{32}P]ATP for 5 min, glucose-1-phosphate, glucose-6-phosphate, G1,6P_2, fructose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, fructose-2,6-bisphosphate, 1,3BPG, and 2,3-bisphosphoglycerate were added, and incubation was allowed to continue for a further 2 min. As shown in Fig. 4, among the agents tested, glucose-1-phosphate caused the most marked effect on removal of ^{32}P from [^{32}P]3PG-PP_{72},

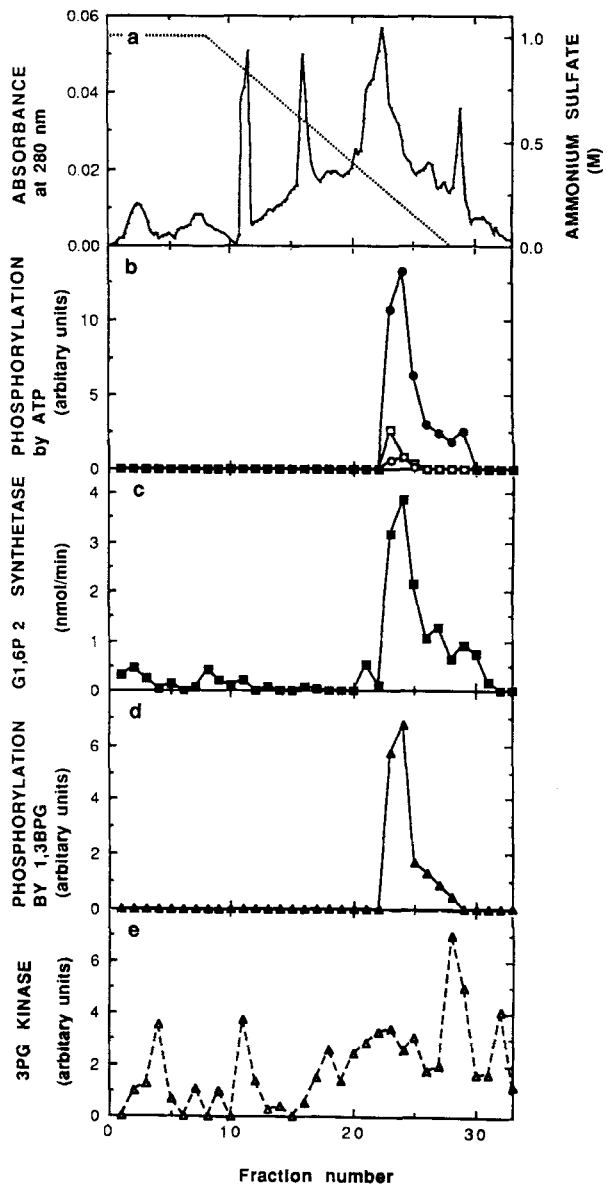

FIG. 2. Phenyl 5PW hydrophobic interaction HPLC of 3PG-PP_{72} and G1,6P_2 synthetase. **a:** Profiles of absorbance at 280 nm and the change of ammonium sulfate concentration. **b:** Phosphorylation of 3PG-PP_{72} by ATP. An aliquot of each fraction (35 μl) was incubated with [γ-^{32}P]ATP in the absence (○) and presence (□) of 3PG or in the presence of 3PG plus exogenous 3PG-dependent kinase (2 μl; ●) for 5 min. ^{32}P incorporation into 3PG-PP_{72} was estimated as described in Fig. 1. **c:** Activity of G1,6P_2 synthetase. An aliquot (50 μl) was used. **d:** Phosphorylation of 3PG-PP_{72} by [1-^{32}P]1,3BPG. An aliquot (50 μl) was incubated with [1-^{32}P]1,3BPG (10 μ*M*). ^{32}P incorporation into 3PG-PP_{72} was estimated as described in Fig. 1. **e:** Activity of "3PG-dependent kinase." Fractions 23 and 24 were combined and aliquots (45 μl) were used as a substrate for "3PG-dependent kinase." Aliquots (5 μl) of each fraction were added as an enzyme source to the incubation mixture, which was then incubated for 90 s. ^{32}P incorporation into 3PG-PP_{72} was detected only when both 3PG and the substrate fraction were added to the incubation mixture.

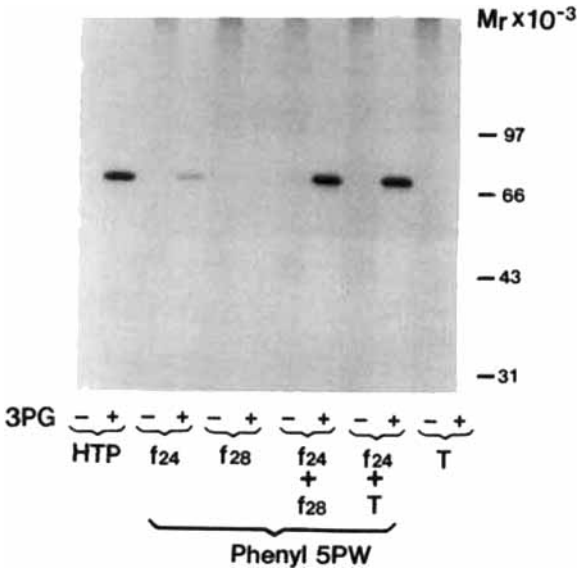

FIG. 3. Separation of 3PG-PP_{72} from kinase. The 3PG-PP_{72} fraction from hydroxyapatite chromatography (HTP; 7.2 μg protein), fraction 24 from Phenyl 5PW HPLC (f_{24}; 0.13 μg 3PG-PP_{72}), fraction 28 from Phenyl 5PW HPLC (f_{28}; 37 μl), fraction 24 and fraction 28 (f_{24} and f_{28}), fraction 24 and Triton X-100 extract of the rat brain microsome fraction (f_{24} + T), and Triton X-100 extract (T; 2 μl) were each incubated in the absence and presence of 3PG for 3 min and subjected to SDS-PAGE and autoradiography. Actual counts incorporated: HTP + 3PG, 384 cpm; f_{24} + 3PG, 76 cpm; f_{24} + f_{28} + 3PG, 460 cpm; f_{24} + T + 3PG, 488 cpm; others are <30 cpm.

followed by glucose-6-phosphate. Presumably, in each case, [^{32}P]G1,$6P_2$ was formed as a result of this removal. These effects were similar to those observed on dephosphorylation of G1,$6P_2$ synthetase (Rose et al., 1977). G1,$6P_2$ and 1,3BPG also showed quite significant effects. The other compounds were much less effective.

Phosphopeptide mapping and phosphoamino acid analysis. To determine whether the site of phosphorylation of 3PG-PP_{72} observed in the presence of 3PG and [γ-^{32}P]ATP is the same as that observed with [1-^{32}P]1,3BPG, 3PG-PP_{72} phosphorylated by both means was subjected to ^{32}P-phosphopeptide and ^{32}P-phosphoamino acid analyses. Phosphorylated 3PG-PP_{72} was digested with (a) trypsin/chymotrypsin, (b) thermolysin, and (c) *Staphylococcus aureus* V8 protease, and the digests were separated by high-voltage thin layer electrophoresis followed by chromatography. Peptide analysis indicates that the major ^{32}P-phosphopeptide obtained with 3PG and [γ-^{32}P]ATP is indistinguishable from that obtained with [1-^{32}P]1,3BPG, regardless of the type of proteolytic enzyme used (Fig. 5).

The trypsin/chymotrypsin digests were subjected to acid hydrolysis to determine the amino acid residues phosphorylated. As shown in Fig. 6, phosphorylation either with [γ-^{32}P]ATP (in the presence of 3PG) or with [1-^{32}P]1,3BPG yielded [^{32}P]phosphoserine. This, together with the data in Fig. 5, strongly suggests that the major site of phosphorylation of 3PG-PP_{72} with [γ-^{32}P]ATP and 3PG is identical to that phosphorylated with [1-^{32}P]1,3BPG.

Stoichiometry of ^{32}P incorporated into 3PG-PP_{72}

After aliquots of the 3PG-PP_{72} fractions from hydroxyapatite chromatography were incubated with 3PG and [γ-^{32}P]ATP or [1-^{32}P]1,3BPG, ^{32}P incorporation into 3PG-PP_{72} was measured (Fig. 7). ^{32}P incorporation from [γ-^{32}P]ATP increased gradually to reach a maximal level at 10 min, while ^{32}P was rapidly incorporated from [1-^{32}P]1,3BPG, reaching a maximal level at 15 s. The values of these maximal levels were 0.141 and 0.169 mol phosphate/mol 3PG-PP_{72}, respectively. Our values are similar to those obtained for G1,$6P_2$ synthetase where the purified enzyme was reacted with [1-^{32}P]1,3BPG in the absence of glucose-1-phosphate (which acts as an acceptor of the 1-position phosphate from 1,3BPG) to yield a value of 0.154–0.231 mol phosphate/mol enzyme (Wong and Rose, 1976).

Evidence for 1,3BPG as immediate donor of phosphate

1,3BPG served as a phosphate donor for the phosphorylation of 3PG-PP_{72} (Figs. 1, 2, and 7). Although 3PG-PP_{72} was labeled with [1-^{32}P]1,3BPG in the absence of ATP (Figs. 1, 2, and 7), it was not known whether 1,3BPG served as the immediate phosphate donor for the phosphorylation of 3PG-PP_{72} that was observed with 3PG and [γ-^{32}P]ATP. To address this issue, three experiments were carried out. First, the effect of unlabeled 1,3BPG on the phosphorylation of

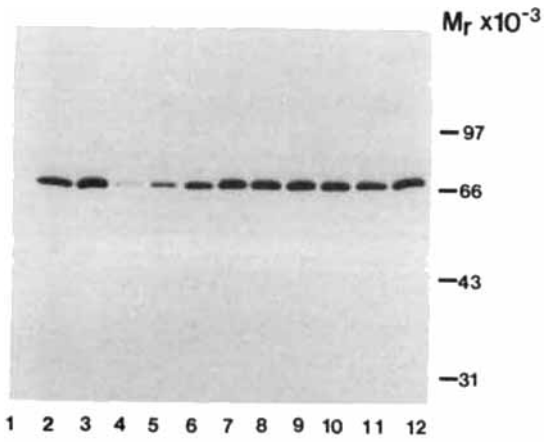

FIG. 4. Effect of glycolytic intermediates on dephosphorylation. The 3PG-PP_{72} fraction from hydroxyapatite chromatography (containing 0.15 μg of 3PG-PP_{72}, 17.0 μg of total protein) was incubated with [γ-^{32}P]ATP in the absence (lane 1) and presence (lane 2) of 3PG for 5 min. After the incubation with [γ-^{32}P]ATP and 3PG for 5 min, various compounds (1 m*M*) were added, and incubation was allowed to continue for an additional 2 min. Lane 3, water; lane 4, glucose-1-phosphate; lane 5, glucose-6-phosphate; lane 6, G1,$6P_2$; lane 7, fructose-1-phosphate; lane 8, fructose-6-phosphate; lane 9, fructose-1,6-bisphosphate; lane 10, fructose-2,6-bisphosphate; lane 11, 1,3BPG; and lane 12, 2,3-bisphosphoglycerate. Samples were subjected to SDS-PAGE and autoradiography. Radioactivities associated with 3PG-PP_{72} in lanes 1–12 were 12, 1,483, 1,970, 312, 636, 1,134, 1,718, 1,736, 1,747, 1,577, 1,319, and 1,736 cpm, respectively.

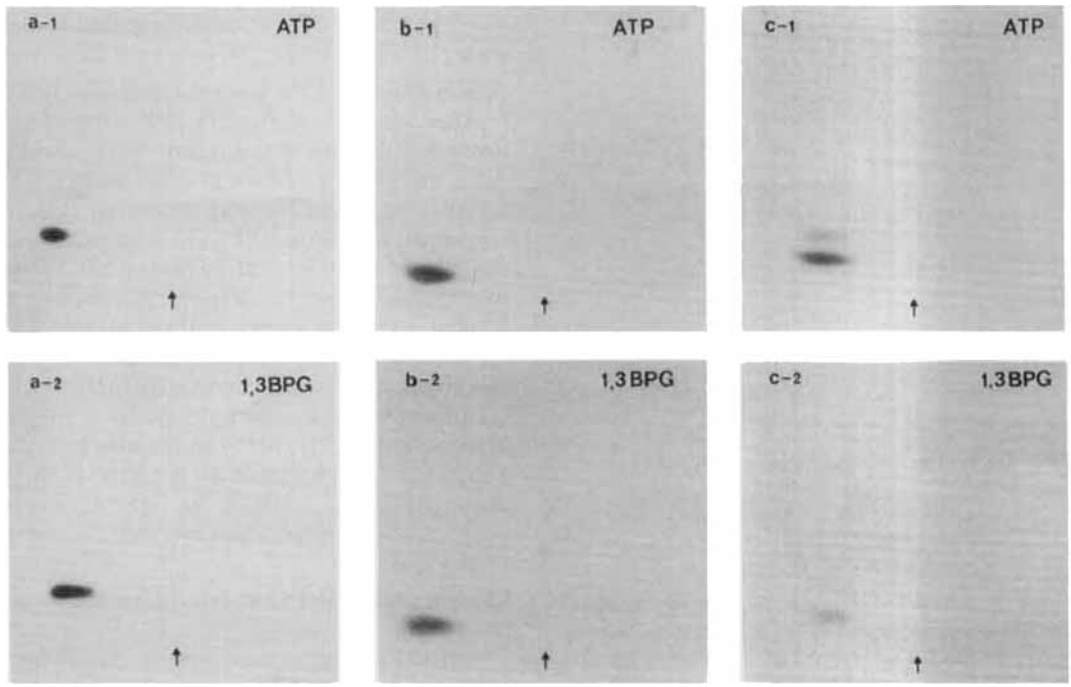

FIG. 5. Peptide mapping after limited proteolysis of phosphorylated 3PG-PP_{72}. The 3PG-PP_{72} fraction from Phenyl 5PW HPLC (containing 0.90 μg of 3PG-PP_{72}) was incubated with (1) [γ-^{32}P]ATP and 3PG or (2) [1-^{32}P]1,3BPG and subjected to SDS-PAGE and autoradiography. When the 3PG-PP_{72} fraction was incubated with [γ-^{32}P]ATP and 3PG, an aliquot of fraction 28 from Phenyl 5PW HPLC was added as a kinase source to the incubation mixture. The dry gel pieces containing 3PG-PP_{72} were reswollen and digested at 37°C for 24 h with trypsin/chymotrypsin (**a-1, a-2**), thermolysin (**b-1, b-2**), and *Staphylococcus aureus* V8 protease (**c-1, c-2**). The digests were separated first by electrophoresis in the horizontal dimension and then by ascending chromatography in the vertical dimension, as described in Materials and Methods. The origin of sample is indicated by arrows; the cathode is on the left and the anode on the right on each plate.

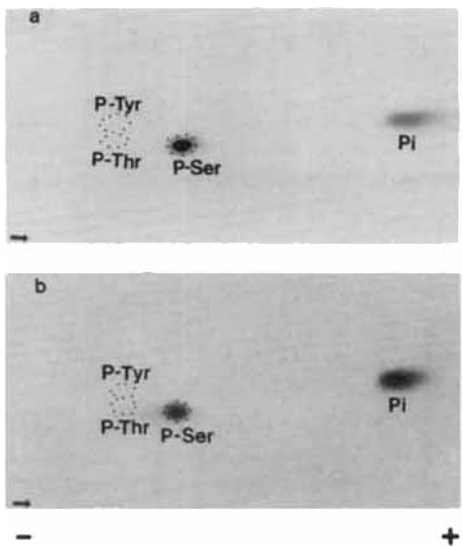

FIG. 6. Identification of the amino acids phosphorylated in 3PG-PP_{72}. The 3PG-PP_{72} fraction from Phenyl 5PW HPLC (containing 0.90 μg of 3PG-PP_{72}) was incubated with [γ-^{32}P]ATP and 3PG (**a**) or [1-^{32}P]1,3BPG (**b**) and subjected to SDS-PAGE and autoradiography. The trypsin/chymotrypsin digests of [^{32}P]3PG-PP_{72} were prepared as described in Fig. 5 and subjected to partial acid hydrolysis. The hydrolysates were analyzed for phosphoamino acids by electrophoresis (in the horizontal dimension) and ascending chromatography (in the vertical dimension). The origin of sample is indicated by arrows.

3PG-PP_{72} with [γ-^{32}P]ATP and 3PG was examined. In data not shown, 1,3BPG effectively reduced the labeling of 3PG-PP_{72}; 50% inhibition was achieved at 10 n*M*. Second, we determined the amount of 1,3BPG generated in a reaction mixture containing [γ-^{32}P]ATP,

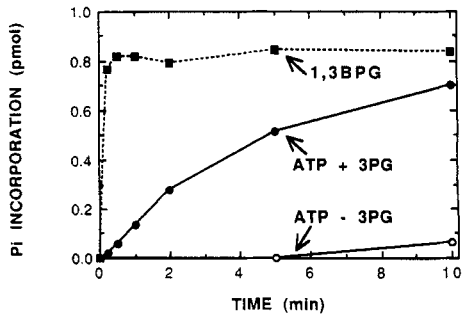

FIG. 7. Stoichiometry of ^{32}P incorporated into 3PG-PP_{72}. The 3PG-PP_{72} fraction from hydroxyapatite chromatography (containing 0.35 μg of 3PG-PP_{72}, 46 μg of total protein) was incubated with [γ-^{32}P]ATP in the presence (ATP + 3PG) and in the absence (ATP − 3PG) of 3PG. It was also incubated with [1-^{32}P]1,3BPG (1,3BPG). Samples were subjected to SDS-PAGE and autoradiography. The bands of [^{32}P]3PG-PP_{72} were cut out and counted in a liquid scintillation spectrophotometer. The amount of 3PG-PP_{72} was determined by densitometric tracing of protein bands stained by Coomassie Brilliant Blue R on slab SDS-PAGE, using bovine serum albumin as standard.

3PG, 3PG-PP_{72}, and our kinase fraction. A significant amount of radioactive 1,3BPG was indeed produced and the concentration of 1,3BPG was calculated to be 83 n*M*. Third, the effectiveness of radioactive 1,3BPG on phosphorylating 3PG-PP_{72} was determined. Figure 8 shows that 3PG-PP_{72} is almost fully phosphorylated with 83 n*M* 1,3BPG; the maximal phosphorylation was achieved at 100 n*M*, whereas the half-maximal phosphorylation was observed at 1.1 n*M*. All of these results support the argument that 1,3BPG serves as the immediate phosphate donor for the phosphorylation of 3PG-PP_{72} observed in the presence of [γ-^{32}P]ATP, 3PG, and 3PG kinase.

Involvement of a distinct protein kinase in phosphorylation of 3PG-PP_{155}

We have previously reported that another protein in the synaptosomal cytosol, referred to as 3PG-PP_{155}, was also phosphorylated in the presence of [γ-^{32}P]ATP and 3PG (Ueda and Plagens, 1987). To determine whether or not this phosphorylation involves 1,3BPG, the synaptosomal cytosol fraction was incubated with [1-^{32}P]1,3BPG. As shown in Fig. 9, in contrast to the phosphorylation of 3PG-PP_{72}, the phosphorylation of 3PG-PP_{155} in the presence of 3PG and ATP was not mimicked by [1-^{32}P]1,3BPG (Fig. 9).

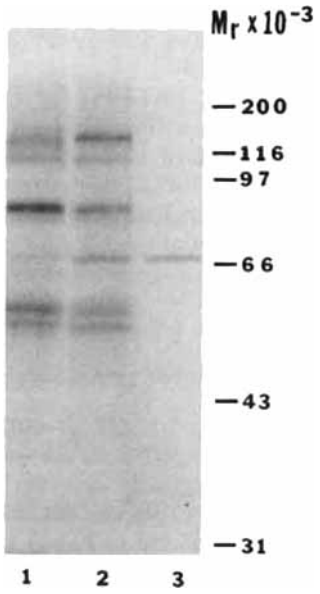

FIG. 9. Protein phosphorylation pattern of synaptosomal cytosol produced with [1-^{32}P]1,3BPG in comparison with that observed with [γ-^{32}P]ATP plus 3PG. Aliquots of the synaptosomal cytosol fraction (25 μg of protein) that had been dialyzed for 18 h against 10 m*M* Tris-HCl buffer (pH 7.4) were phosphorylated with 20 μ*M* [γ-^{32}P]ATP (16.5 Ci/mmol) for 2 min in the absence (lane 1) and presence (lane 2) of 5 m*M* 3PG or with 10 μ*M* [1-^{32}P]1,3BPG (10.6 Ci/mmol; lane 3) in the absence of 3PG. The phosphorylated samples were then subjected to SDS-PAGE and autoradiography.

DISCUSSION

In this study, we have demonstrated that the 3PG-dependent protein phosphorylation system for 3PG-PP_{72} was resolved into two components, the substrate 3PG-PP_{72} and a kinase, which is likely to be 3PG kinase catalyzing the formation of 1,3BPG from 3PG and ATP. Several lines of evidence indicate that 3PG-PP_{72} is G1,6P_2 synthetase. These include (a) the identical chromatographic behavior we observed for both on hydroxyapatite and hydrophobic interaction chromatographies, (b) the ability of 1,3BPG to serve as the direct phosphate donor for the phosphorylation of both, (c) the identical amino acid phosphorylated by 1,3BPG or by ATP and 3PG in the presence of 3PG-dependent kinase, (d) highly similar phosphopeptide fragments generated with proteolytic enzymes, (e) highly similar molecular weights in SDS, and (f) the abundant occurrence of both in the mammalian brain. Thus, it is likely that the phosphorylation of 3PG-PP_{72} with [γ-^{32}P]ATP observed in the presence of 3PG in the synaptosomal cytosol fraction represents the phosphoryl transfer to 3PG-PP_{72} from the acyl phosphate of 1,3BPG, which is generated from 3PG and ATP, perhaps by 3PG kinase present in the preparation.

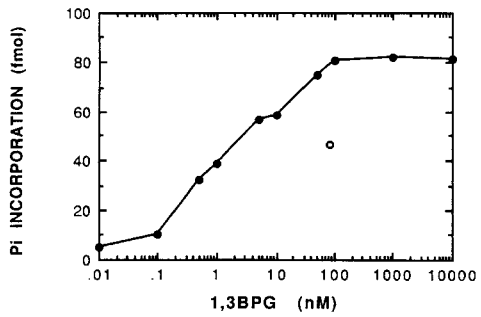

FIG. 8. Effect of various concentrations of [1-^{32}P]1,3BPG on phosphorylation of 3PG-PP_{72}. The 3PG-PP_{72} fraction from Phenyl 5PW HPLC (containing 0.14 μg of 3PG-PP_{72}) was incubated with various concentrations of [1-^{32}P]1,3BPG for 2 min (●). Samples were subjected to SDS-PAGE and autoradiography. The gel pieces containing 3PG-PP_{72} were cut out, using the autoradiogram as a guide, and the amount of $^{32}P_i$ incorporated was determined in a liquid scintillation spectrophotometer. The 3PG-PP_{72} fraction from Phenyl 5PW HPLC was also incubated with 20 μ*M* [γ-^{32}P]ATP and 5 m*M* 3PG in the presence of the major kinase fraction [fraction 28, 37-μl aliquot (○)].

In both our study and that of Rose et al. (1977), the value calculated for the amount of ^{32}P incorporated into G1,6P_2 synthetase is too low if the enzyme is homogeneous. However, it is likely that the homogeneous protein may contain either an inactive or a phosphorylated form of the enzyme.

It is known that 1,3BPG serves as a phosphoryl donor not only to G1,6P_2 synthetase (Rose et al., 1975) but also to 2,3-bisphosphoglycerate synthetase-phosphatase (Rose and Whalen, 1973). Whereas 1,3BPG phosphorylates a serine residue of G1,6P_2 synthetase (Wong and Rose, 1976), it phosphorylates a histidine residue on 2,3-bisphosphoglycerate synthetase-phosphatase (Rose and Whalen, 1973). G1,6P_2 synthetase has a molecular weight of 72,000 and is a monomer (Wong and Rose, 1976), while the subunit of 2,3-bis-

phosphoglycerate synthetase-phosphatase has a molecular weight of 30,000 in SDS-PAGE and is a dimer (Rose and Whalen, 1973).

The apparent K_m of 3PG-PP_{72} for 1,3BPG in the phosphorylation reaction (Eq. 1) is 1.1 nM. On the other hand, a K_m value of 0.07 μM was reported by Wong and Rose (1976) for G1,$6P_2$ synthetase, as measured in the overall synthetase reaction in the presence of an infinite amount of glucose-1-phosphate, i.e., the rate of G1,$6P_2$ formation (Eqs. 1 and 2):

$$1,3BPG + E \rightarrow E\text{-}P + 3PG \quad (1)$$

$$E\text{-}P + \text{glucose-1-P} \rightarrow G1,6P_2 + E \quad (2)$$

The low apparent K_m, 1.1 nM or 0.07 μM, of the G1,$6P_2$ synthetase for 1,3BPG in the formation of E-P may reflect physiologically relevant levels of the 1,3BPG. The levels of 1,3BPG in the brain and erythrocytes have been estimated to be <1 μM (Lowry et al., 1964) and 0.39–0.7 μM (Minakami and Yoshikawa, 1965), respectively.

G1,$6P_2$, the product of the G1,$6P_2$ synthetase reaction, is a phosphate donor to a family of enzymes that participate in unique sugar phosphate mutase reactions, such as those not only for glucose phosphate but also for ribose phosphate, mannose phosphate, and acetylglucosamine phosphate (Rose, 1986). G1,$6P_2$ can also act as a regulator of some of the key enzymes in carbohydrate metabolism. It has been shown to activate phosphoglucomutase, phosphofructokinase, and pyruvate kinase and to inhibit hexokinase, 6-phosphogluconate dehydrogenase, and fructose-1,6-diphosphatase (Beitner, 1985). Since 1,3BPG is the phosphate donor for the formation of G1,$6P_2$, 1,3BPG could serve as a feed-forward regulator in glycolysis.

The regional distribution of G1,$6P_2$ synthetase in the brain coincides with that of G1,$6P_2$ and IMP-dependent G1,$6P_2$ phosphatase (Yip et al., 1985). However, it does not parallel the distribution of phosphoglucomutase, and G1,$6P_2$ levels are much higher than the concentration required for the cofactor role in the phosphoglucomutase reaction (Yip et al., 1988). In addition, the G1,$6P_2$ system failed to exhibit a regional correlation with other enzymes involved in carbohydrate metabolism and with glucose consumption (Sokoloff et al., 1977). Moreover, 3PG-PP_{72}, now identified as G1,$6P_2$ synthetase, has been shown to be present in the brain at much higher concentrations than in "nonnervous tissues" such as liver, kidney, lung, and heart (Ueda and Plagens, 1987). Based upon these observations, the possibility is raised that G1,$6P_2$ may fill some other function besides serving as the cofactor for sugar phosphate mutase. For example, it may serve as a phosphate donor for a neuronal protein and thereby play a role in synaptic function. The results presented here and by Rose et al. (1975) suggest that G1,$6P_2$ production is facilitated through an increase in the formation of the active phosphorylated intermediate of G1,$6P_2$ synthetase by the glycolytic intermediate 1,3BPG. Thus, it is conceivable that glycolysis could play a role in synaptic function via 1,3BPG and G1,$6P_2$ followed by G1,$6P_2$-supported phosphorylation of a synaptic protein.

In contrast to the phosphorylation system for 3PG-PP_{72}, where the 72-kDa protein is phosphorylated by either ATP and 3PG or 1,3BPG, the 155-kDa protein is phosphorylated by ATP and 3PG, but not by 1,3BPG. Direct transfer of phosphate from ATP to the protein substrate rather than via the intermediate 1,3BPG formation remains a viable possibility and is compatible with the notion that a 3PG-dependent protein kinase is responsible for the phosphorylation of the 155-kDa protein. Thus, the 155-kDa protein is phosphorylated in a manner different than is 3PG-PP_{72}. This notion is also supported by the previous observation that, unlike the phosphorylation of 3PG-PP_{72}, the phosphorylation of 3PG-PP_{155} with [γ-^{32}P]ATP was stimulated not only by 3PG but also by unlabeled 2-phosphoglycerate or 2,3-bisphosphoglycerate (Ueda and Plagens, 1987).

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