

# Identification of the 64 Kilodalton Chloroplast Stromal Phosphoprotein as Phosphoglucomutase<sup>1</sup>

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## ABSTRACT

Phosphorylation of the 64 kilodalton stromal phosphoprotein by incubation of pea (*Pisum sativum*) chloroplast extracts with [ $\gamma$ -<sup>32</sup>P]ATP decreased in the presence of Glc-6-P and Glc-1,6-P<sub>2</sub>, but was stimulated by glucose. Two-dimensional gel electrophoresis following incubation of intact chloroplasts and stromal extracts with [ $\gamma$ -<sup>32</sup>P]ATP, or incubation of stromal extracts and partially purified phosphoglucomutase (EC 2.7.5.1) with [<sup>32</sup>P]Glc-1-P showed that the identical 64 kilodalton polypeptide was labeled. A 62 kilodalton polypeptide was phosphorylated by incubation of tobacco (*Nicotiana sylvestris*) stromal extracts with either [ $\gamma$ -<sup>32</sup>P]ATP or [<sup>32</sup>P]Glc-1-P. In contrast, an analogous polypeptide was not phosphorylated in extracts from a tobacco mutant deficient in plastid phosphoglucomutase activity. The results indicate that the 64 (or 62) kilodalton chloroplast stromal phosphoprotein is phosphoglucomutase.

Phosphorylation of chloroplast proteins is well documented, particularly for polypeptides associated with the thylakoid membranes (2). The best understood of the chloroplast phosphoproteins in terms of identity and function are the 23 and 25 kD apoproteins of the light-harvesting Chl *a/b* complex (1, 2). Several soluble proteins in the chloroplast stroma also are phosphorylated on incubation of intact chloroplasts with [ $\gamma$ -<sup>32</sup>P]ATP or [<sup>32</sup>P]Pi. The most heavily labeled of the stromal phosphoproteins is a polypeptide with a relative molecular mass of between 64 and 70 kD, depending upon the species (2, 3, 5, 8, 9, 11, 18). Phosphorylation of this polypeptide has been observed in intact chloroplasts (2, 8, 11), in soluble extracts prepared from lysed chloroplasts (3, 5, 11, 18), and in mixed membrane preparations that contain the inner membrane and outer envelope of the chloroplast plus the interluminal material between these membranes (18, 19).

<sup>32</sup>P incorporation into the 64 to 70 kD polypeptide (henceforth referred to as the 64 kD polypeptide) exhibits rapid turnover during pulse-chase experiments (18, 19) and is inhibited *in vitro* by ADP (5, 19). The 64 kD polypeptide is phosphorylated on a serine residue (3, 5) by a mechanism which, unlike the light-harvesting Chl *a/b* proteins (1), is

insensitive to changes in redox state. For example, <sup>32</sup>P-labeling of the 64 kD polypeptide is unaffected by dark/light transitions (8, 11), the presence of photosynthesis inhibitors (3, 5, 11), or exposure to anaerobic conditions (3). These labeling characteristics have led to the general conclusion that the 64 kD polypeptide is phosphorylated by a unique, redox-independent stromal kinase (3, 5, 9).

In the present study, the effects of various metabolites on phosphorylation of the 64 kD stromal polypeptide from pea chloroplasts and a 62 kD polypeptide from tobacco chloroplasts were examined and the phosphoproteins were compared with those of a tobacco mutant deficient in PGM<sup>2</sup> activity. From the results of these experiments, it is concluded that phosphorylation of the 64 kD chloroplast stromal polypeptide can be ascribed mainly to autophosphorylation of plastid phosphoglucomutase.

## MATERIALS AND METHODS

### Biochemicals

[ $\gamma$ -<sup>32</sup>P]ATP was synthesized from [<sup>32</sup>P]Pi (ICN Biomedicals<sup>3</sup>) by a modified exchange reaction (14). [<sup>32</sup>P]Glc-1-P was isolated as a by-product from the synthesis of 5-N<sub>3</sub>UDP-Glc (7).

### Plant Material and Chloroplast Isolation

Intact chloroplasts were isolated from darkened leaves of pea (*Pisum sativa* L. var Little Marvel) and a diploid tobacco species, *Nicotiana sylvestris* (Spegazzini and Comes) (12). The 'starchless' *N. sylvestris* mutant (NS 458), which contains a defective plastid PGM, has been described previously (10). Chloroplast stromal extracts were prepared by hypotonic lysis of intact chloroplasts at 4°C. Intact chloroplasts were suspended at 10  $\mu$ g Chl/mL in 20 mM Tricine-KOH (pH 7.9), 5 mM MgCl<sub>2</sub>, and 4 mM DTT (buffer A). Following centrifugation to remove the thylakoid membranes, the soluble extract was concentrated 15-fold by ultrafiltration on an Amicon

<sup>2</sup> Abbreviations: PGM, phosphoglucomutase; IEF, isoelectric focusing; PGA, 3-phosphoglyceric acid.

<sup>3</sup> Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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YM-30 membrane before use in the assay. Soluble protein in the extracts was determined by the Coomassie dye binding assay (Bio-Rad).

### Phosphorylation Assays

Phosphorylation of proteins *in vitro* was performed by incubating stromal extracts for 6 min at 25°C with 0.2 mM [ $\gamma$ - $^{32}$ P]ATP or [ $^{32}$ P]Glc-1-P in buffer A. Additions to the assays are indicated in the text. Phosphorylation of proteins *in organello* was performed by incubating intact chloroplasts (100  $\mu$ g Chl/mL) at 25°C with 0.4 mM [ $\gamma$ - $^{32}$ P]ATP in isotonic buffer containing 4.0 mM PPI (17). Chloroplasts were irradiated with white light at 1000  $\mu$ mol photons/m<sup>2</sup>·s during the 6 min incubation. Chloroplasts were pelleted by low speed centrifugation and the stromal extract was isolated following lysis in hypotonic buffer. Total proteins in the stromal extracts were separated by SDS-PAGE on 10 to 18% polyacrylamide gels and the phosphorylated proteins were visualized by autoradiography. Two-dimensional (IEF  $\times$  SDS-PAGE) gel electrophoresis was performed as previously described (6).

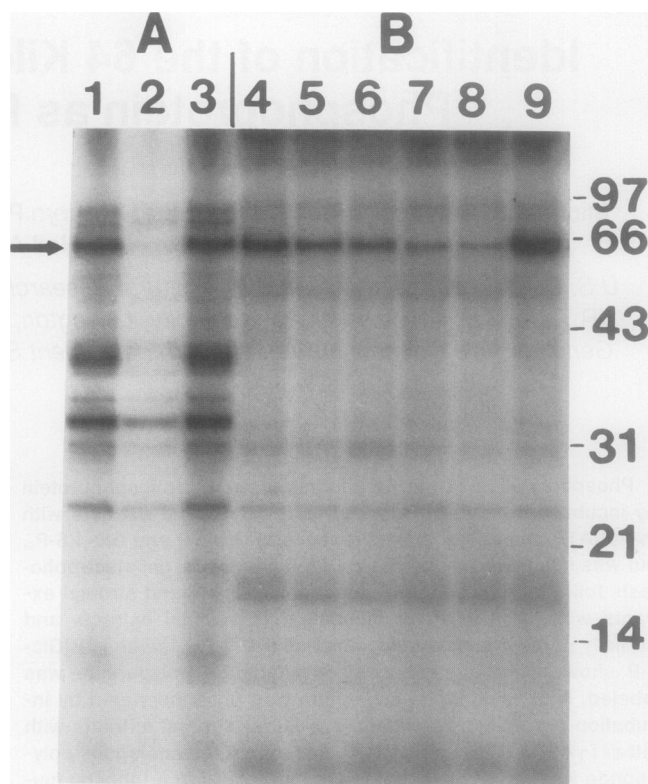
### Purification of Phosphoglucomutase

Phosphoglucomutase was partially purified from stromal extracts of pea chloroplasts by chromatography on a 10 mL column of DEAE-cellulose. The column was eluted with a 0 to 0.5 M linear KCl gradient. Active fractions were pooled and ultrafiltration was used to change the buffer to 20 mM potassium phosphate (pH 7), 1 mM EDTA and 4 mM DTT. The concentrated material was applied to a 5 mL hydroxyapatite column and fractions containing phosphoglucomutase activity, which eluted with 70 mM potassium phosphate, were concentrated by ultrafiltration in buffer A. The final preparation, which was purified approximately 10-fold, was stored frozen at -80°C. Phosphoglucomutase activity was determined by monitoring NADPH production in an assay coupled to Glc-6-P dehydrogenase (15).

## RESULTS

### Phosphorylation of the 64 kD Polypeptide *in Vitro* and *in Organello*

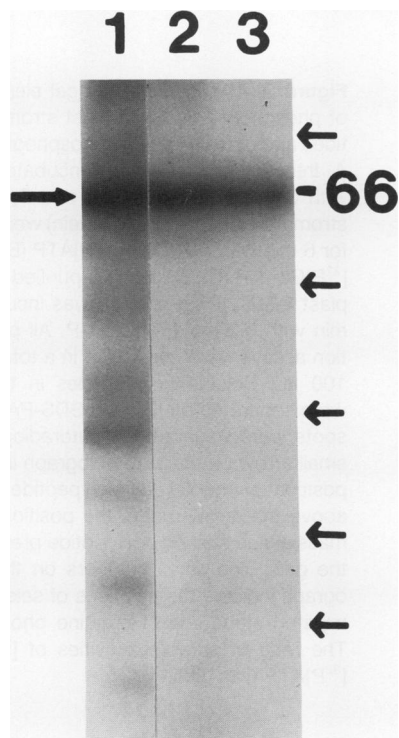
Several polypeptides in the chloroplast stroma were phosphorylated when intact pea chloroplasts or chloroplast stromal extracts were incubated with [ $\gamma$ - $^{32}$ P]ATP (Fig. 1). As reported previously (2, 5, 8, 11), one of the most heavily labeled of the stromal phosphoproteins was a 64 kD polypeptide. Phosphorylation of this polypeptide *in organello* was almost completely eliminated by the addition of 2 mM 3-phosphoglyceric acid (PGA), but was increased in the presence of 2 mM glucose (Fig. 1). In contrast, PGA or PGA plus PGA kinase had only a slight inhibitory effect on phosphorylation of the 64 kD polypeptide *in vitro* in a chloroplast stromal extract (Fig. 1). However, phosphorylation of this polypeptide in the stromal extract was reduced markedly in the presence of 160  $\mu$ M Glc-1,6-P<sub>2</sub>, both in the presence and absence of PGA. The addition of glucose to the stromal extract caused a marked increase in phosphorylation of the 64 kD polypeptide (Fig. 1).



**Figure 1.** Phosphorylation of the 64 kD polypeptide in intact and lysed pea chloroplasts. A, Intact chloroplasts were incubated for 6 min with 0.4 mM [ $\gamma$ - $^{32}$ P]ATP either alone (lane 1) or in the presence of 2 mM PGA (lane 2) or 2 mM glucose (lane 3) in a total volume of 100  $\mu$ L; B, chloroplast stromal extracts were incubated for 6 min with 0.2 mM [ $\gamma$ - $^{32}$ P]ATP either alone (lane 4) or in the presence of 2 mM PGA (lane 5), 2 mM PGA plus 3 units/mL PGA kinase (lane 6), 160  $\mu$ M Glc-1,6-P<sub>2</sub> (lane 7), 2 mM PGA and 160  $\mu$ M Glc-1,6-P<sub>2</sub> (lane 8), or 2 mM glucose (lane 9) in a total volume of 25  $\mu$ L. Soluble polypeptides were separated by SDS-PAGE and the bands were visualized by autoradiography. The arrow indicates the position of the 64 kD polypeptide. Numbers in the right-hand margin refer to the positions and relative molecular mass of standard proteins.

### Phosphorylation of Chloroplastic PGM

The opposite effects of unlabeled glucose and Glc-1,6-P<sub>2</sub> on phosphorylation of the 64 kD polypeptide in chloroplast stromal extracts suggested the possibility that the 64 kD polypeptide was phosphorylated via hexokinase-generated Glc-6-P rather than by direct phosphoryl transfer from  $\gamma$ -labeled ATP. Autophosphorylation by Glc-P is reminiscent of the  $^{32}$ P-labeling pattern observed with phosphoglucomutase, a 62 to 70 kD phosphoprotein which exchanges phosphate with Glc-6-P or Glc-1-P in the course of the reaction sequence (15). To determine the relationship between the 64 kD stromal phosphoprotein and PGM, PGM was partially purified from pea chloroplasts and incubated in the phosphorylation assay with [ $\gamma$ - $^{32}$ P]ATP plus glucose and hexokinase or with [ $^{32}$ P]Glc-1-P. In Figure 2, phosphorylation of partially purified pea plastid PGM is compared with phosphorylation of polypeptides in stromal extracts that were incubated with [ $\gamma$ - $^{32}$ P]ATP. With partially purified PGM, a



**Figure 2.** Phosphorylation of the 64 kD polypeptide and partially purified phosphoglucumutase from pea chloroplasts. Lane 1, Chloroplast stromal extracts (30  $\mu$ g protein) were incubated for 6 min with 0.2 mM [ $\gamma$ - $^{32}$ P]ATP in a total volume of 25  $\mu$ L as described in Figure 1; lanes 2 and 3, partially purified PGM from pea chloroplasts (10  $\mu$ g protein) was incubated for 6 min with either 0.2 mM [ $\gamma$ - $^{32}$ P]ATP, 2 mM glucose, and 2 units/mL hexokinase (lane 2) or with 0.2 mM [ $^{32}$ P]Glc-1-P (lane 3) in a total volume of 25  $\mu$ L. Polypeptides were separated by SDS-PAGE and the bands were visualized by autoradiography. The left-hand arrow indicates the position of the 64 kD polypeptide. The arrows and number in the right-hand margin refer to the positions of the relative molecular mass standard proteins in Figure 1. The ratio of specific activities of [ $^{32}$ P]Glc-1-P/[ $^{32}$ P]ATP was 0.65.

single polypeptide corresponding to the 64 kD stromal phosphoprotein was phosphorylated by incubation with either [ $^{32}$ P]Glc-6-P, formed *in situ* from [ $\gamma$ - $^{32}$ P]ATP plus glucose and hexokinase, or with [ $^{32}$ P]Glc-1-P (Fig. 2). Phosphorylation of partially purified PGM by [ $^{32}$ P]Glc-1-P decreased markedly in the presence of 2 mM Glc-6-P (data not shown).

#### Two-Dimensional Gel Electrophoresis of the 64 kD Polypeptide and PGM

The polypeptides labeled by incubation of intact pea chloroplasts or chloroplast stromal extracts with [ $\gamma$ - $^{32}$ P]ATP, or by incubation of chloroplast stromal extracts or partially purified stromal PGM with [ $^{32}$ P]Glc-1-P were separated by two-dimensional (IEF  $\times$  SDS-PAGE) gel electrophoresis (Fig. 3, A–D). A similar two-dimensional profile of  $^{32}$ P incorporation was observed at 64 kD in all cases, regardless of the source of the  $^{32}$ P label (*i.e.* Glc-1-P *versus* ATP) or the type of preparation (*i.e.* intact chloroplasts, stromal extracts or

partially purified PGM). The profile of radioactivity at 64 kD was characterized by distribution of label in three regions of the IEF dimension; two spots near the acidic end which contained most of the label, a faint region consisting of several diffuse spots at a slightly more basic pH, and a diffuse region closer to the basic end of the gel. The diffuse region in the intact chloroplast samples appeared at a more basic position relative to its position in the other samples (Fig. 3A). All of the 64 kD isoforms were labeled most heavily in the preparation of partially purified PGM (Fig. 3D).

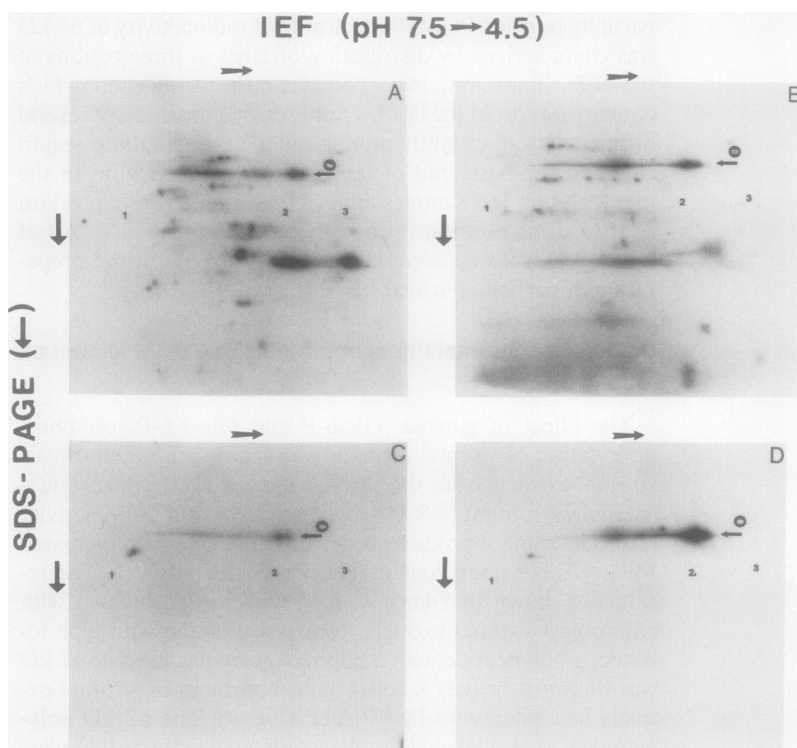
#### Chloroplast Stromal Phosphoproteins in a PGM Mutant of Tobacco

The effect of glucose, Glc-6-P and Glc-1,6-P<sub>2</sub> on phosphorylation of stromal proteins was examined in chloroplast stromal extracts from the tobacco species *N. sylvestris* and a 'starchless' mutant (NS 458) deficient in plastid PGM activity (10). The mutant produces a defective enzyme with increased Michaelis constants and probably a decreased  $V_{max}$ . The results are shown in Figure 4 along with those obtained with chloroplast stromal extracts from pea. For the wild-type tobacco, a polypeptide with a relative molecular mass of 62 kD was the most heavily labeled phosphoprotein in stromal extracts incubated with [ $\gamma$ - $^{32}$ P]ATP (Fig. 4). The 62 kD polypeptide was also labeled in tobacco stromal extracts that were incubated with [ $^{32}$ P]Glc-1-P (data not shown). For both the 62 kD polypeptide in tobacco and the 64 kD polypeptide in pea, phosphorylation was reduced markedly in the presence of Glc-1,6-P<sub>2</sub> and Glc-6-P, but was increased in the presence of glucose. Phosphorylation of a 62 kD polypeptide was not detectable in stromal extracts from the tobacco PGM mutant, even when extracts from the mutant were incubated with [ $^{32}$ P]Glc-1-P (data not shown) or with [ $\gamma$ - $^{32}$ P]ATP in the presence of glucose (Fig. 4). The failure to detect a 62 kD labeled polypeptide in the mutant was consistent with the low rate of  $^{32}$ P-labeling expected for a PGM enzyme with such unfavorable  $K_m$ (Glc-P) values (10).

#### DISCUSSION

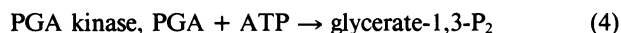
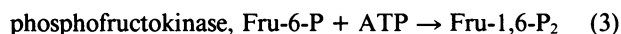
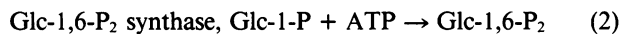
Three lines of evidence indicate that the 64 kD stromal phosphoprotein in pea chloroplasts is PGM. First, phosphorylation of the 64 kD polypeptide by incubation of stromal extracts with [ $\gamma$ - $^{32}$ P]ATP was reduced markedly by Glc-6-P and Glc-1,6-P<sub>2</sub> a substrate and cofactor, respectively, for PGM. Second, phosphorylation of the 64 kD polypeptide occurred when stromal extracts were incubated with [ $^{32}$ P]Glc-1-P. Phosphorylation of the 64 kD polypeptide by [ $^{32}$ P]Glc-1-P produced an IEF profile similar to the profile produced by incubation with [ $\gamma$ - $^{32}$ P]ATP and was similar to the more heavily labeled profile produced by incubating partially purified PGM with [ $^{32}$ P]Glc-1-P. Third, phosphorylation of an analogous 62 kD stromal polypeptide in tobacco was not observed in stromal extracts from a tobacco mutant that contained a defective plastid PGM.

PGM, which is present in plants as cytosolic and plastidic isozymes (13), catalyzes the intramolecular phosphate transfer between the 1 and 6 positions of glucose (15). An obligatory step in the PGM reaction mechanism is the transfer of phos-



**Figure 3.** Two-dimensional gel electrophoresis of phosphorylated chloroplast stromal polypeptides and partially purified phosphoglucumutase. A, Intact chloroplasts were incubated for 6 min with 0.4 mM [ $\gamma$ - $^{32}$ P]ATP; B and C, chloroplast stromal extracts (122  $\mu$ g protein) were incubated for 6 min with 0.2 mM [ $\gamma$ - $^{32}$ P]ATP (B) or 0.2 mM [ $^{32}$ P]Glc-1-P (C); D, partially purified pea chloroplast PGM (40  $\mu$ g protein) was incubated for 6 min with 0.2 mM [ $^{32}$ P]Glc-1-P. All phosphorylation assays were conducted in a total volume of 100  $\mu$ L. Soluble polypeptides in the extracts were separated by IEF and SDS-PAGE and the spots were visualized by autoradiography. The small arrow on the autoradiograph indicates the position of the 64 kD polypeptide. The circle above the arrow marks the position of a Coomassie blue stained polypeptide present in all of the gels. The small numbers on the autoradiograph indicate the positions of selected carbamylated standards of creatine phosphokinase. The ratio of specific activities of [ $^{32}$ P]Glc-1-P/[ $^{32}$ P]ATP was 0.65.

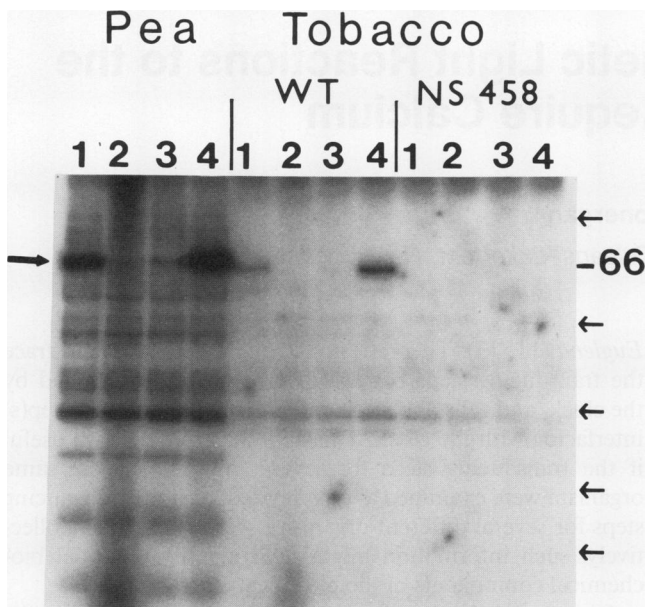
phate from the serine of the phosphoenzyme to the supplied substrate (Glc-6-P or Glc-1-P) to form dephosphorylated enzyme and Glc-1,6-P<sub>2</sub>. Generally, Glc-1,6-P<sub>2</sub> does not dissociate but reacts again with enzyme to yield product (Glc-6-P or Glc-1-P) and phosphoenzyme, in which the original phosphate on the enzyme is replaced by that of the substrate (15). Alternate substrates for the reaction include fructose-P and most hexose-P and glycerate-1,3-P<sub>2</sub> can replace Glc-1,6-P<sub>2</sub> as a cofactor of PGM (15). Thus, when [ $\gamma$ - $^{32}$ P]ATP is added to crude extracts, PGM may be phosphorylated not by ATP directly, but possibly by one or more of the labeled intermediates generated by the following one-step reactions:



In the present study, the stimulatory effects of glucose on phosphorylation of the 64 kD polypeptide indicated that phosphorylation of plastid PGM by [ $\gamma$ - $^{32}$ P]ATP probably occurs primarily via the hexokinase reaction. Phosphorylation of pea plastid PGM via [ $^{32}$ P]glycerate-1,3-P<sub>2</sub> did not appear to be a major factor contributing to the phosphorylation of plastid PGM, since phosphorylation of the 64 kD polypeptide was not increased in the presence of PGA. Presumably, there is sufficient Glc-1,6-P<sub>2</sub> bound to PGM in chloroplasts and in stromal extracts to prevent glycerate-1,3-P<sub>2</sub> from serving as a cofactor of PGM in these crude systems. Rather, PGA inhibited PGM phosphorylation, particularly in the intact chloroplast system. We attribute this inhibition to inhibition of the hexokinase reaction (4) caused by elevated ADP levels (16).

Characteristic features of the 64 kD phosphorylation reaction such as its insensitivity to redox state (3, 5, 8, 11), inhibition by ADP (5, 19), rapid turnover (18, 19), and high potential in vesicular systems (18) are all consistent with intramolecular autophosphorylation of PGM by its substrate. That is, those factors that affect phosphorylation of the 64 kD polypeptide also influence the concentrations or synthesis of the PGM substrate, Glc-P. Thus, changes in the extent of phosphorylation of the 64 kD polypeptide under a variety of conditions, which were previously attributed to the properties of a unique protein kinase can be explained more readily by changes in the availability of labeled PGM substrates.

The nearly complete inhibition of phosphorylation that occurred in the presence of unlabeled Glc-6-P appeared to rule out the possibility that 64 kD phosphoprotein in chloroplast extracts is composed of other functionally distinct phosphoproteins besides PGM. In a previous study, Soll and Bennett (18) used limited proteolytic digestion to demonstrate that the 64 kD chloroplast phosphoprotein labeled in stromal extracts was identical to the 64 kD polypeptide labeled in mixed membrane fractions. In the present study, two-dimensional gel electrophoresis showed that 64 kD phosphoprotein is primarily one functional species, PGM, by demonstrating that similar IEF profiles were obtained with intact chloroplasts incubated with [ $\gamma$ - $^{32}$ P]ATP, stromal extracts incubated with [ $\gamma$ - $^{32}$ P]ATP and [ $^{32}$ P]Glc-1-P and partially purified PGM labeled with [ $^{32}$ P]Glc-1-P. Phosphorylation assays with chloroplast stromal extracts from a well-characterized tobacco mutant containing a defective plastid PGM (10) also support this point by demonstrating that phosphorylation of the analogous 62 kD chloroplast phosphoprotein was undetectable in the PGM mutant. Taken together, the genetic and biochem-



**Figure 4.** Phosphorylation of the 64 and 64 kD polypeptide in stromal extracts from pea and tobacco chloroplast and the absence of labeling in extracts from a PGM-deficient tobacco mutant. Chloroplast stromal extracts from pea, tobacco wild-type (WT), and the PGM-deficient mutant (NS 458) were incubated for 6 min with 0.2 mM [ $\gamma$ - $^{32}$ P]ATP either alone (lane 1) or in the presence of 2 mM Glc-6-P (lane 2), 160  $\mu$ M Glc-1,6-P<sub>2</sub> (lane 3), or 2 mM glucose (lane 4) in a total volume of 25  $\mu$ L. Soluble polypeptides were separated by SDS-PAGE and the bands visualized by autoradiography. The left-hand arrow indicates the position of the 64 kD polypeptide. The arrows and number in the right-hand margin refer to the positions of the relative molecular mass standard proteins in Figure 1.

ical evidence indicate that the pea 64 kD and the tobacco 62 kD stromal phosphoproteins are PGM, and that they are phosphorylated, not by a unique protein kinase, but by autophosphorylation in the course of the reaction with Glc-6-P.

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