Articles

Affinity Labeling and Purification of Spinach Leaf Ribulose-5-phosphate Kinase†

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ABSTRACT: Spinach leaf ribulose-5-phosphate kinase has been purified to homogeneity by a procedure incorporating affinity chromatography. The purified enzyme requires a divalent cation for activity and has a specific activity of 360 units/mg. It is composed of two apparently identical subunits. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicates a subunit M_r of 45 000. The enzyme is inactivated by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine in a site-directed fashion. The reaction is pseudo first order both in the presence and absence of Mg^{2+}. The presence of Mg^{2+} retards the nonspecific loss of activity in the absence of the affinity label while accelerating the rate of inactivation by the affinity label. In the presence of Mg^{2+}, K_i = 4.8 mM and k_{inact} = 4.22 min^{-1} at 30 °C. The rate of inactivation is slightly accelerated by the presence of ribulose 5-phosphate. While Mg^{2+}-ADP and Mg^{2+}-ATP offer some protection, the greatest protection is provided by Mg^{2+}-ADP–sugar phosphate complexes. The inactivation is largely reversible with dithiothreitol, thus suggesting the modification of an active site cysteine residue.

Ribulose-5-phosphate kinase (EC 2.7.1.19) catalyzes the synthesis of the photosynthetic CO_2 acceptor ribulose 1,5-bisphosphate (eq 1).[1] Activity of the bacterial enzyme is

$$\text{ribulose 5-phosphate} + \text{ATP} \xrightarrow{M^{2+}} \text{ribulose 1,5-bisphosphate} + \text{ADP} \quad (1)$$

subject to regulation by allosteric effectors (Tabita, 1980; Siebert et al., 1981). The plant enzyme is subject to inhibition by various stromal metabolites, but these effects can be explained by competition for the substrate binding site (Flugge et al., 1982; Gardemann et al., 1983). Additionally, the activity of the enzyme is modulated by a thioredoxin-mediated disulfide exchange process (Latzko et al., 1970; Wolosiuk & Buchanan, 1978).

Previously reported work on the spinach enzyme was performed with chloroplast extracts or partially purified enzyme. Detailed mechanistic studies of the enzyme would be facilitated by the availability of a fully purified enzyme. Several methods for purification of the spinach enzyme have appeared in the literature; however, the purity of these preparations has not been clearly established (Hurwitz et al., 1956; Racker, 1957; Slabas & Walker, 1976). Electrophoretically pure Ru5P kinase has been obtained by an extensive modification of the method of Lavergne and Bismuth (1973), including an affinity chromatography procedure to produce a preparation that is free of contamination by ribulosebisphosphate carboxylase, ribose-5-phosphate isomerase, and ATPase activities. Enzyme prepared in this fashion has been used previously to demonstrate the stereochemical course of the reaction (Miziorko & Eckstein, 1984a,b). The availability of a relatively stable, high-purity preparation has facilitated characterization and affinity labeling of the enzyme. This paper indicates how the affinity labeling approach can be used as an effective tool for investigating the binary and ternary complexes formed by the enzyme. A preliminary account of this work has appeared (Krieger & Miziorko, 1985).

Experimental Procedures

Materials

DTT was obtained from Boehringer. [2-^{14}C]-5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine ([^{14}C]FSBA) was obtained from New England Nuclear. ATP–agarose (attached through C-8 with a six-carbon spacer) and unlabeled 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) were obtained from Sigma. Reagents for protein determination were obtained from Bio-Rad. All other biochemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods

Enzyme Assays. All assays were performed at 30 °C. The coupled assay contains 10 mM $MgCl_2$, 5 mM ATP, 5 mM ribose 5-phosphate, 0.2 mM PEP, 0.2 mM NADH, 0.5 mM DTT, 2 units of pyruvate kinase, 1 unit of lactate dehydrogenase, and 1 unit of phosphoriboisomerase in 100 mM Tris-HCl buffer at pH 8.2 (Racker, 1957). Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Purification of Ribulose-5-phosphate Kinase. Unless otherwise stated, all steps were carried out at 0–4 °C. Fresh, deveined spinach leaves (660 g) were suspended in two 1-L portions of 10 mM potassium phosphate buffer, pH 7.6/0.1 mM EDTA/10 mM 2-mercaptoethanol. The leaves were homogenized for 1 min at medium speed in a Waring blender. The resulting mixture was then filtered through four layers

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; DMF, *N*,*N*-dimethylformamide; DTT, dithiothreitol; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; OAc, acetate; RuBP, ribulose 1,5-bisphosphate; Ru5P, ribulose 5-phosphate; PEP, phosphoenolpyruvate; 6-PG, 6-phosphogluconate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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of two-ply cheesecloth. The dark, green brei was then subjected to a 37% ammonium sulfate fractionation by slowly adding solid ammonium sulfate. The precipitate was removed by centrifugation at 14000*g* for 15 min.

The pale green supernatant was cooled to −10 °C, and 0.8 volume of precooled acetone (dry ice) was added over a 10-min period with continuous stirring. The addition was quite exothermic, but the mixing solution was maintained below 2 °C throughout the addition. The resulting suspension was stirred for 15 min at −10 °C. The suspension was centrifuged in polyethylene bottles for 10 min at 14000*g*. The supernatant was discarded, and the pellets were dried under a stream of N_2. The pellets are resuspended in 660 mL (1 mL/g of leaf) of 100 mM Tris-HCl, pH 8.0/10 mM EDTA/0.5 mM DTT with a large Potter-Elvehjem homogenizer at 300 rpm. The suspension was stirred an additional 15 min and then centrifuged (14000*g*) for 15 min and the pellet discarded.

The pH of the supernatant was lowered to 5.0 by the gradual addition of 1 M potassium acetate, pH 4.5. The resulting suspension was stirred for 15 min and then centrifuged for 15 min (14000*g*). After the pellets were discarded, the pH of the supernatant was rapidly raised to 7.0 by adding 1 M potassium phosphate, pH 7.5.

The enzyme was precipitated by adding solid ammonium sulfate to a final concentration of 55%. After centrifugation (14000*g*, 15 min), the pellets were dissolved in 50 mL of 5 mM potassium phosphate buffer, pH 7.0/5 mM $MgCl_2$/0.1 mM EDTA/0.5 mM DTT. The protein solution was dialyzed twice vs. 4-L portions of the same buffer. The dialyzate was loaded onto a hydroxylapatite column (5 × 10 cm) equilibrated with the same buffer. The column was eluted in a 1-L gradient with the potassium phosphate concentration going from 5 to 100 mM at a flow rate of 1 mL/min. The enzyme elutes as a single peak beginning at approximately 55 mM potassium phosphate. The active fractions were then concentrated to a final volume of 16 mL in an Amicon ultrafiltration cell. If the enzyme was to be stored at this point, glycerol was added to a final concentration of 20%. The solution was stored frozen at −70 °C.

A portion of the concentrated enzyme (1500–2000 units) was dialyzed vs. 2-L portions of 10 mM potassium phosphate buffer, pH 7.0/0.1 mM EDTA/0.5 mM DTT for 8 h with a buffer change. The dialyzate was loaded onto a 5-mL column of ATP–agarose at a flow rate of 5 mL/h. The column was washed with the same buffer until the A_{280} was less than 0.1. The column was eluted with 10 mM potassium phosphate, pH 7.0/10 mM ATP/0.1 mM EDTA/0.5 mM DTT at a flow rate of 0.5 mL/h. Fractions of 1.0 mL were collected. Under these conditions, most of the ribulose-5-phosphate kinase activity is recovered in a 3-mL volume. Typically, one hydroxylapatite column yields sufficient activity for three ATP–agarose affinity chromatography steps. The yield reported for the ATP–agarose step in the purification table is based on the enzyme recovered from three affinity column elutions.

A summary of the purification appears in Table I. The procedure yields more than a 600-fold purification and approximately 20% yield in terms of activity.

Molecular Weight Determination. The subunit molecular weight was determined by SDS–PAGE. The samples were dissolved in a solution containing 8 M urea, 1% SDS, and 5% 2-mercaptoethanol and boiled for 3 min. The samples were then electrophoresed on a 10% polyacrylamide gel containing 50 mM sodium phosphate, 0.1% SDS, and 4 M urea. The molecular weight markers used were bovine serum albumin (65000), RuBP carboxylase, large subunit (55000), ovalbumin (45000), carbonic anhydrase (30000), lactoglobulin (18400), and RuBP carboxylase, small subunit (15000).

Inactivation with 5′-[p-(Fluorosulfonyl)benzoyl]adenosine. The affinity-purified enzyme, 30 μL at 1.0 mg/mL, was activated by adding 10 μL of 100 mM DTT and incubating at 30 °C for 1 h. After activation, 100 μL of 50 mM potassium veronal buffer at pH 7.9 was added, and the activated enzyme was kept at 0 °C.

The inactivation solutions (0.4 mL) were prepared by adding 5 μL of the activated enzyme to a solution containing potassium veronal, pH 7.9, and potassium chloride (final concentrations were 25 and 100 mM, respectively). The final concentration of DTT due to carry-over was 90 μM. This level does not significantly reverse the modification. The inactivation was initiated by adding 60 μL of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine dissolved in DMF. The FSBA solutions were prepared fresh daily, and the concentrations were determined by the absorbance at 259 nm with an extinction coefficient of 15800 M^{-1} cm^{-1} (Pal et al., 1975). The inactivations were conducted at 30 °C. The progress of the reaction was monitored by assaying aliquots spectrophotometrically.

Stoichiometry of Labeling. Ribulose-5-phosphate kinase (1 mg, 1 mg/mL) was dialyzed vs. 1 L of 10 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA and 0.5 mM DTT. The enzyme was activated by adding DTT to a final concentration of 25 mM and incubating at 30 °C for 1 h. The DTT level was reduced to less than 20 μM by repeated concentration in an Amicon centrifugal concentrator and dilution with 25 mM potassium veronal, pH 7.9. The enzyme solution (850 μL) was mixed with 150 μL of [^{14}C]FSBA (1.9 mM final concentration; 6.5 × 10^6 cpm/μmol) to initiate inactivation. Inactivation was carried out at 30 °C. The inactivation was followed by diluting 5-μL aliquots into 1.25 mL of 25 mM potassium veronal, pH 7.9, and assaying spectrophotometrically until less than 1.0% of the initial activity remained. Since phosphate buffer has been demonstrated to accelerate hydrolysis of the sulfonyl fluoride (Wyatt & Colman, 1977), the reaction mixture is diluted to 1.25 mL by adding 1 M potassium phosphate, pH 7.5, at 0 °C. The sample is concentrated to 50 μL and diluted to 1 mL with potassium phosphate buffer (100 mM) before dialysis to eliminate excess active reagent. The sample is then exhaustively dialyzed vs. 10 mM potassium phosphate, pH 6.8, to remove any remaining excess.

Incorporation of [^{14}C]FSBA with Time. Ru5P kinase (1 mg) was dialyzed, activated, and washed to reduce the DTT level as described above. The enzyme was diluted to 5 mL by the addition of 50 mM potassium veronal, pH 7.9. This was followed by the addition of 1 mL of 1 M KCl, 100 μL of 1 M $MgCl_2$, and 2.4 mL of water, yielding a volume of 8.5 mL. The inactivation was initiated by the addition of 1.5 mL of 2 mM [^{14}C]FSBA (5140 cpm/nmol). The inactivation was followed by assaying aliquots spectrophotometrically. Incorporation of radiolabel was followed by removing 500-μL aliquots and adding them to 9 mL of ice-cold acetone. The precipitated protein was collected by filtration through Whatman GF/A glass fiber filters. The collected precipitate was washed 15 times with 5-mL portions of cold acetone to remove unbound label.

RESULTS

Enzyme Purification and Characterization. Table I presents a summary of the purification steps. One of the major obstacles in purifying spinach enzymes is the elimination of ribulose 1,5-bisphosphate carboxylase from the preparation.

Table I: Purification of Spinach Ribulose-5-phosphate Kinase

step	units	mg of protein	sp act.	purification (x-fold)
brei	9480	15 700[a]	0.60	1
37% ammonium sulfate supernatant	8600	7830	1.10	1.8
acetone precipitation	8500	733	11.6	19
pH 5.0 treatment	7500	423	17.7	30
55% ammonium sulfate precipitate	5720	265	21.6	36
hydroxylapatite column	4900	67	73.1	122
ATP–agarose column[b]	2700	7.5	360	600

[a] This number is probably a high estimate due to colored contaminants. [b] Combined yield of three ATP–agarose columns.

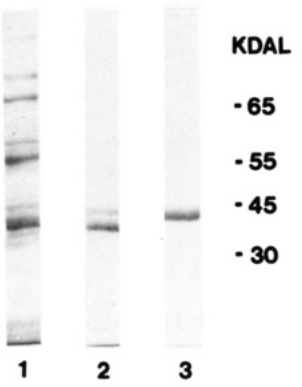

FIGURE 1: SDS–polyacrylamide gel electrophoresis of Ru5P kinase after various steps in the purification procedure. SDS–PAGE was performed with a 4.5% stacking gel and a 10% separating gel according to the method of Laemmli (1970). Molecular weight markers used were bovine serum albumin (65 kdaltons), RuBP carboxylase, large subunit (55 kdaltons), ovalbumin (45 kdaltons), and carbonic anhydrase (30 kdaltons). (Lane 1) Acetone precipitation; (lane 2) hydroxylapatite pool; (lane 3) ATP–agarose fraction. Each lane contained 5 μg of protein.

The carboxylase represents the major portion of protein in the initial spinach extract. While the acetone precipitation removes a significant portion of the carboxylase, further treatment is necessary to quantitatively eliminate the carboxylase from the preparation. Lowering the pH to 5.0 precipitates most of the remaining RuBP carboxylase while allowing recovery of the Ru5P kinase activity in excellent yield. As can be seen in Figure 1, the carboxylase is quantitatively removed before the affinity chromatography step is implemented. The hydroxylapatite column was found to be necessary before the affinity chromatography step. Ribulose-5-phosphate kinase elutes in a single peak midway through the gradient and is preceded by a small shoulder. Despite the elimination of over 95% of the initial protein, the kinase remains a minor component of the preparation at this point. Chromatography on ATP–agarose not only completes the purification procedure but also concentrates the enzyme activity to a 3-mL volume. The peak appears after 6–7 mL of the ATP containing buffer has been passed through the column. The principal enzyme-containing fractions are quite homogeneous. The reported yield does not include the trailing fractions, which are more dilute and are frequently contaminated by minor impurities. Initially, Mg^{2+} was included in the elution buffer but was found to have no beneficial effects. The purified kinase is stored by adding glycerol to a final concentration of 20% and freezing at −70 °C. Enzyme stored in this fashion slowly loses activity over several months and can be conveniently reactivated by incubation with DTT. If the affinity-purified enzyme is reactivated in the presence of millimolar levels of ATP, the reactivation

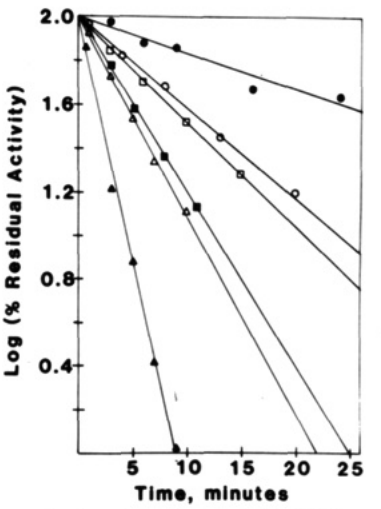

FIGURE 2: Concentration dependence of Ru5P kinase inactivation by FSBA. All inactivations were conducted at 30 °C in 25 mM potassium veronal, pH 7.9, containing 10 mM $MgCl_2$, 100 mM KCl, and 15% DMF. Inactivations were initiated by adding a solution of FSBA in DMF. Aliquots were removed at time points and immediately assayed in the standard assay. The concentrations of FSBA were 0 (●), 0.756 (▲), 0.216 (△), 0.169 (■), 0.091 (□), and 0.070 mM (○).

is time-dependent, with incubation in the presence of 25 mM DTT at 30 °C for 1 h or 50 mM DTT at 30 °C for 15 min yielding maximally active enzyme. If the ATP level is reduced by dialysis or dilution, the enzyme is fully active after incubation with 25 mM DTT for 2 min at 30 °C. The lower rate of reaction with DTT in the presence of ATP has been noted by other workers (Omnaas et al., 1985). Attempts to reactivate the enzyme with DTT levels of 15 mM or less resulted in only a partial increase in enzyme activity. The freshly purified enzyme and the reactivated enzyme have specific activities between 350 and 370 units/mg. The enzyme requires the presence of divalent cations for activity. Mg^{2+} and Mn^{2+} have been shown to be active. The reactivated enzyme can be diluted and is stable for at least 15 h at 0 °C.

Subunit molecular weights were determined by electrophoresis on SDS–urea gels. When electrophoresis is conducted by the method of Laemmli (1970), the ribulose-5-phosphate kinase band migrates slightly faster than the ovalbumin marker. This appears to be caused by the retention of some tertiary structure. The last vestiges of the tertiary structure can be eliminated by denaturation and electrophoresis in a phosphate-buffered gel (pH 7.0) containing 4 M urea in addition to 0.1% SDS. This yields a single band with a molecular weight of 45 000 per subunit, which agrees with a value previously reported (Lavergne & Bismuth, 1973). Chromatography of the native enzyme on Sephacryl 200 yields a single peak, which elutes at approximately 90 000 (data not shown). These results indicate that the kinase is composed of two subunits, in accordance with the report of Kagawa (1982) for the tobacco leaf enzyme.

Affinity Labeling with FSBA. Figure 2 shows the loss of activity in the presence of FSBA. The rate of inactivation is dependent on the concentration of FSBA and exhibits pseudo-first-order kinetics. Assuming the mechanism of inactivation involves initial formation of a reversible enzyme–inhibitor binary complex followed by covalent modification, the reaction can be expressed as in eq 2. The observed rate of

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E^*I \xrightarrow{k_3} E\text{–}I \qquad (2)$$

inactivation at a given inhibitor concentration can be deter-

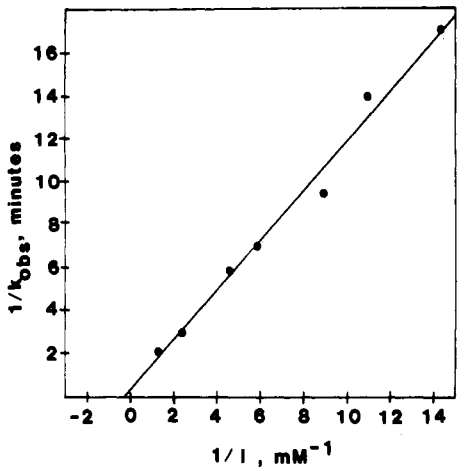

FIGURE 3: Determination of K_i and k_{inact} for FSBA. The values for k_{obsd} were determined by plotting ln (specific activity) vs. time. The slopes are equal to $k_{obsd} + k_0$, where k_0 is the rate of enzyme inactivation in the absence of the affinity label. The k_{obsd} values were calculated by subtracting the rate of inactivation in the absence of affinity label from the rate of inactivation in the presence of the label at the concentrations depicted in Figure 2.

Table II: [(Fluorosulfonyl)benzoyl]adenosine Inactivation of Ru5P Kinase: Protection by Binary Complexes[a]

sample	relative rate of inactivation
Mg^{2+}	100
no additions	59.1
ATP (1.7 mM)	39.9
Mg^{2+}, ATP (0.87 mM)	94.6
Mg^{2+}, ATP (6.6 mM)	51.3
Mg^{2+}, ADP (2.0 mM)	73.8
Mg^{2+}, Ru5P (0.87 mM)	109
-FSBA	23.0
Mg^{2+}, -FSBA	13.0

[a] All samples were incubated at 30 °C in 25 mM potassium veronal, pH 7.9, containing 100 mM KCl and 15% DMF. Inactivations were initiated by adding FSBA in DMF to yield a final concentration of 0.49 mM unless otherwise stated. When present, $MgCl_2$ was added to give a total Mg^{2+} concentration of 10 mM.

mined from a plot of ln (specific activity) vs. time. The slope is k_{obsd}. Since

$$k_{obsd} = k_3[I]/(K_i + [I]) \quad (3)$$

and

$$1/k_{obsd} = (K_i/k_3)(1/[I]) + 1/k_3 \quad (4)$$

a replot of $1/k_{obsd}$ vs. $1/[I]$ will yield K_i and k_3 (Figure 3). This yields $K_i = 4.8$ mM and $k_3 = 4.22$ min^{-1}, where $k_3 = k_{inact}$ (estimated as $[I] \rightarrow \infty$).

The presence of Mg^{2+} has a distinct effect upon the inactivation Ru5P kinase. The presence of Mg^{2+} appears to reduce the rate of nonspecific enzyme inactivation in the absence of FSBA (Table II). Additionally, the site-directed inactivation by FSBA is accelerated when Mg^{2+} is present. The enzyme is partially protected against FSBA inactivation by ATP in the absence of Mg^{2+}; however, little protection is provided by Mg^{2+}-ATP in the presence of Mg^{2+} unless very high concentrations of Mg^{2+}-ATP are used in the experiment. In the absence of Mg^{2+}, increasing the level of ATP present during affinity labeling results in a decrease in the rate of enzyme inactivation (Figure 4) as expected if ATP and FSBA compete for the same binding site. No protection is offered by ribulose 5-phosphate. In fact, the addition of saturating Ru5P consistently increases the rate of inactivation by a small amount (10%).

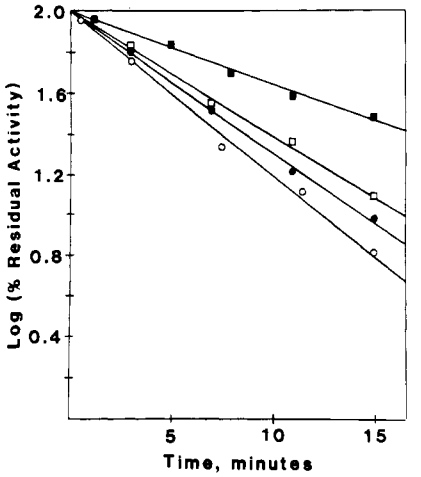

FIGURE 4: Concentration dependence of ATP protection against FSBA inactivation. All inactivations were conducted at 30 °C in 25 mM potassium veronal, pH 7.9, containing 100 mM KCl and 15% DMF. The final concentration of FSBA was 200 μM. Inactivations were initiated by adding a solution of FSBA in DMF. Aliquots were removed at time points and immediately assayed in the standard assay. The concentrations of ATP were 0 (○), 0.328 (●), 0.735 (□), and 1.47 mM (■).

Table III: [(Fluorosulfonyl)benzoyl]adenosine Inactivation of Ru5P Kinase: Protection by Occupancy of Nucleotide and Sugar Phosphate Sites[a]

sample	relative rate of inactivation
$MgCl_2$, KCl	100
$MgCl_2$, ADP, Ru5P (0.78 mM), KCl	47.3
$Mg(NO_3)_2$, ADP, Ru5P (0.78 mM), KNO_3	46.5
$Mg(OAc)_2$, ADP, Ru5P (0.78 mM), KOAc	43.6
$MgCl_2$, ADP, 6-PG (2.7 mM), KCl	32.4
$MgCl_2$, ADP, RuBP (4.0 mM), KCl	20.8

[a] All inactivations were conducted at 30 °C in 25 mM potassium veronal, pH 7.9, containing 15% DMF. Inactivations were initiated by adding FSBA in DMF to a final concentration of 0.49 mM. The concentrations of magnesium and potassium salts were 10 and 100 mM, respectively. When present, the total ADP concentration was 2.0 mM.

The rate of inactivation by FSBA is only reduced by approximately 25% in the presence of Mg^{2+}-ADP. Protection under these conditions is increased by the addition of a sugar phosphate (Table III). The rate of inactivation by FSBA is reduced by 50% when Ru5P is added in conjunction with Mg^{2+}-ADP. The degree of protection provided is unaffected by the substitution of nitrate or acetate ions for chloride ions in the reaction mixture. Greater protection is obtained when 6-phosphogluconate, a competitive inhibitor (Anderson, 1973; Gardemann et al., 1983), is used. The best protection is obtained with the product complex of Mg^{2+}-ADP–RuBP. Under these circumstances, the rate of FSBA-dependent inactivation is reduced to approximately twice the slow rate of the activity decay of the untreated enzyme.

The enzyme is not inactivated by phenylmethanesulfonyl fluoride. Additionally, incubation of the modified enzyme (99.5% of the initial activity eliminated) with 50 mM DTT at 30 °C results in the recovery of 87% of the original activity within 20 min. This suggests that cysteine is the site of modification. Inactivation (less than 1% of the initial activity remaining) of the kinase with [^{14}C]FSBA results in the incorporation of 0.93 mol of [^{14}C]FSBA/mol of enzyme subunit. The inactivation of the enzyme closely parallels the incorporation of [^{14}C]FSBA (Figure 5). The specificity of modification indicated by these data as well as the excellent protection afforded by the product complex, Mg^{2+}-ADP–RuBP,

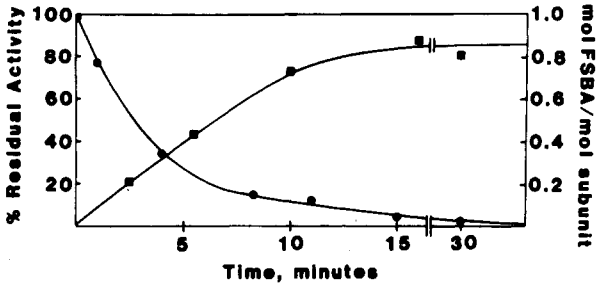

FIGURE 5: Correlation of incorporation of [^{14}C]FSBA with extent of enzyme inactivation. The inactivation was conducted at 30 °C in 25 mM potassium veronal, pH 7.9, containing 10 mM $MgCl_2$, 100 mM KCl, and 15% DMF. Inactivation was initiated by adding a solution of [^{14}C]FSBA in DMF. The concentration of [^{14}C]FSBA was 300 μM (sp act. = 5140 cpm/nmol). Enzyme activity (●) and ^{14}C incorporation (■) were measured as described under Methods.

suggests that FSBA is acting as an active site directed inhibitor.

DISCUSSION

Spinach ribulose-5-phosphate kinase has been purified to homogeneity in good yield. While other workers have reported the purification of this enzyme, only Lavergne and Bismuth (1973) have presented electrophoretic evidence of purity. In our hands, their procedure did not yield electrophoretically pure enzyme. Similar results have been reported by other researchers (Omnaas et al., 1985). The procedure reported herein has consistently (10 full purifications) yielded homogeneous Ru5P kinase. Additionally, the yield in terms of enzyme units per gram of spinach is significantly higher than that reported previously. The combined acetone precipitation and low-pH treatment provides a fast, effective method for the removal of the prodigious quantity of ribulose-1,5-bisphosphate carboxylase from the preparation. The hydroxylapatite column also removes a significant quantity of contaminating protein. The procedure requires 3 days to reach the affinity chromatography step. The protein obtained from the hydroxylapatite column should be sufficient for loading two to three ATP–agarose columns. The affinity chromatography step yields highly purified enzyme in a concentrated form. The yield of the affinity step typically ranges from 50 to 70% with the lowest yields obtained from columns being used for the first time. The affinity resin can be conveniently regenerated by passing 25–30 mL of buffer containing 1 M KCl through the column followed by equilibration with the loading buffer. The affinity column can be loaded, eluted, and regenerated with a turnaround time of 2 days.

The native enzyme consists of two apparently identical subunits with a M_r of 45 000/subunit. To date, Mg^{2+} and Mn^{2+} have been shown to satisfy the divalent cation requirement. Studies are in progress to determine the role of the metal ion in catalysis. The specific activity of the kinase is 360 units/mg. This high activity (implying a low number of active sites present for the kinase) would make the enzyme an efficient point for controlling the rate of CO_2 fixation. Previous work with chloroplast extracts suggests that the level of ribulose-5-phosphate kinase activity is influenced by the level of stromal metabolites (Gardemann et al., 1983); however, it was not established whether this was caused by direct interaction of these metabolites with the enzyme or occurred through some intermediate process. The purification procedure described will allow the convenient preparation of homogeneous ribulose-5-phosphate kinase. The availability of the pure enzyme makes possible more detailed studies of the kinetics, mechanism, and regulation of this enzyme that may provide a greater insight into the process of CO_2 fixation.

The enzyme is inactivated by FSBA in a site-directed fashion. Partial protection is provided by ATP, high levels of Mg^{2+}-ATP, and Mg^{2+}-ADP. This suggests that the label is interacting with the nucleotide binding site. The failure of Mg^{2+}-ATP to provide good protection at concentrations approaching saturation (0.70 mM) was surprising. One possible explanation is that the enzyme has an ordered, or highly synergistic, mechanism with Ru5P binding first. Research on yeast hexokinase indicates that at low levels of phosphoryl donor, the mechanism is ordered with the acceptor (glucose) binding first; however, this binding order may be overridden at high levels of phosphoryl donor to give a random binding mechanism (Kosow & Rose, 1970, 1971). Alternately, the substrates may bind randomly with the enzyme–Mg^{2+}–Ru5P Michaelis complex having a greater affinity for Mg^{2+}-ATP than the binary enzyme–Mg^{2+} complex. The increased rate of inactivation in the presence of Ru5P alone may reflect these mechanisms.

The interaction of the enzyme with ATP, Mg^{2+}-ATP, Mg^{2+}-ADP, and FSBA in the absence of sugar phosphate may reflect the enzyme's basic affinity for the adenosyl group or for nucleosides in general. Upon sugar phosphate binding, the enzyme may adopt a conformation that enhances the binding of the phosphorylated derivatives, particularly triphosphates. An example of such a phenomenon is provided by crystallographic studies on the isoenzymes of hexokinase, which demonstrate a large conformational change upon binding the phosphoryl acceptor (Bennett & Steitz, 1978; Steitz et al., 1976; McDonald et al., 1979); this change may restructure the nucleotide binding site.

Milner-White and Watts (1971) found that the addition of nitrate ions to product complexes of creatine kinase provided increased protection to inactivation by iodoacetic acid. These researchers postulated that this effect was caused by the planar nitrate anion mimicking the development of a planar phosphate in the transition state. While nitrate ion inhibits the Ru5P kinase reaction (unpublished results), there is essentially no difference in the degree of protection provided by Mg^{2+}-ADP–Ru5P in the presence of nitrate, chloride, or acetate ions. These results may indicate that the three complexes examined have similar stabilities.

The best protection is obtained in the presence of Mg^{2+}-ADP plus 6-phosphogluconate or RuBP. These compounds are similar in that both possess an anionic group that may occupy the region of the active site in which phosphate transfer may occur. Since the Ru5P kinase reaction has been shown to be slowly reversible (Racker, 1957), the protection by the product complex is not unexpected. The reason for protection by the Mg^{2+}-ADP–6-phosphogluconate complex is rather less clear. The phosphogluconate may simply be an analogue of RuBP in the reverse reaction.

The ability of DTT to reverse inhibition suggests that a thiolsulfonate linkage is formed by reaction of the label with a cysteine residue (Annamalai & Colman, 1981; Tomich et al., 1981) since the alkyl sulfonate or sulfonamide linkage formed by reaction with a hydroxyl or an amino group should be stable to this treatment. Modification of an active site cysteine by this demonstrated ATP analogue is especially interesting in light of the results obtained by Hartman's group (Omnaas et al., 1985; Porter & Hartman, 1985) with (bromoacetyl)ethanolamine phosphate. Although their analogue seems similar in structure to ribulose 5-phosphate, ATP provided significant protection against inactivation. After tryptic digestion, a peptide containing a (carboxymethyl)cysteine

residue was isolated, indicating the target of this reagent to be an active site cysteinyl sulfhydryl. It has not been determined whether both FSBA and (bromoacetyl)ethanolamine phosphate modify the same residue. The enzyme's tendency to be oxidatively inactivated also raises the possibility that either affinity label may be modifying a cysteinyl sulfhydryl involved in conversion of the enzyme to the catalytically active species. The inability of oxidatively inactivated Ru5P kinase to incorporate (bromoacetyl)ethanolamine phosphate (Omnaas et al., 1985) supports this possibility. Inactivation of the enzyme by the formation of an intramolecular disulfide near the ATP binding site could render the active site inaccessible or block a catalytically essential sulfhydryl group.

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