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Short Communication

Heavy Metal Impurities Impair the Spectrophotometric Assay of Ribulose Bisphosphate Carboxylase Activity[1]

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ABSTRACT

An inverse relationship between the concentration of ribose 5-phosphate and apparent ribulose bisphosphate carboxylase activity was observed. The Lilley-Walker assay spectrophotometric assay, in which the 3-phosphoglyceric acid-dependent oxidation of reduced pyridine nucleotide is measured, is shown to be highly sensitive to inhibition by heavy metals. Analysis of the purity of reagents showed that ribose 5-phosphate is often contaminated with lead in sufficient quantity to impair the assay. This noncompetitive inhibition by ribose 5-phosphate is independent of the competitive inhibition of this substrate as an ATP sink as described by Slabas and Walker. A method for checking reagent purity and removing heavy metal contaminants is described.

Spectrophotometric determination of RuDPCase[2] activity by measurement of 3PGA-dependent oxidation of NADH is a convenient alternative to the radioisotope assay of $^{14}CO_2$ fixation. An improved spectrophotometric method has been recently described by Lilley and Walker (5) in which an ATP-regenerating system was added to chloroplast extracts to prevent the accumulation of ADP. Transient inhibition of photosynthetic O_2 evolution by rib 5P and ADP has been reported in reconstituted chloroplasts due to the high sensitivity of phosphoglycerate kinase to changes in ATP:ADP ratio (8, 9). Rib 5P is expected to have no direct effect on 3PGA phosphorylation in the absence of chloroplast extract, the source of phosphoribulokinase and ribose phosphate isomerase. However, initial experiments with the spectrophotometric determination of RuDPCase activity indicated that some rib 5P preparations contain an inhibitor of 3PGA metabolism in addition to the transient effect of rib 5P on ATP:ADP ratio in the presence of chloroplast extract. This inhibitory activity is due to heavy metal contamination of some commercial preparations of rib 5P. A method for detecting and removing heavy metal ion impurities from commercial rib 5P was therefore developed.

MATERIALS AND METHODS

Plant Material. Although initial observations were made with chloroplasts isolated from *Panicum maximum* Jacq., results with spinach (*Spinacia oleracea*) are reported here. Ten g of purchased spinach leaves were rinsed for 30 min in cold water in daylight. The leaves were then mixed with 140 ml grinding medium (0.33 M sorbitol, 5 mM $MgCl_2$, 50 mM tris-HCl (pH 8) 0.1% BSA, 0.1 mM mercaptoethanol) and liquified in a Waring Blendor for 2 sec. The liquid was filtered through a 20-μm nylon net, and the filtrate centrifuged at 1000g for 15 min. From the resultant pellets, lyzed chloroplasts were prepared by resuspending pellets in grinding medium lacking sorbitol. Aliquots of lyzed material were removed for Chl determination in 80% acetone (1) and protein determination (6). Lyzed chloroplasts were used within 15 min.

Source of Reagents. All biochemicals were obtained from Sigma Co. Enzyme solutions of creatine phosphokinase (EC 2.7.3.2), 3-phosphoglycerate phosphokinase (EC 2.7.2.3), and NAD-dependent glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) were prepared from stock daily in 0.1 M tris (pH 7.6) with 0.1% fraction V BSA added to promote stability on dilution. Various lots of rib 5P (grade 99-100% pure) were used in these studies. Preweighed vials of NADH and ATP were prepared daily in 10 mM tris (pH 8).

Spectrophotometric Assay of Enzyme Activity. The procedure of Lilley and Walker (5) for the spectrophotometric determination of RuDPCase (EC 4.1.1.39) activity forms the basis of the present study. The principle of the measurement rests on the stoichiometric oxidation of 2 mol NADH/mol CO_2 fixed. NADH oxidation in reaction mixtures was measured by extinction at 340 nm in a Gilford monochrometer equipped with a programmable automatic sample changer, allowing comparison with a freshly prepared NADH standard. The rate of the partial reaction of 3PGA-dependent oxidation of NADH upon addition of exogenous 3PGA was measured at the terminus of each RuDPCase assay to ascertain whether the reaction proceeded with the expected rate and stoichiometry of 1 mol 3PGA added/mol NADH oxidized.

Chromatography of Substrates. Ribose 5-phosphate and other substances were analyzed by TLC on 8-cm Silica Gel G prescored plates (Analtech) in 95% ethanol (60%)/0.1 M ammonium tetraborate (pH 9) (40%). Sugars were visualized using $AgNO_3$-NaOH (ref. 3, reagent No. 234). Chelex-100 (Bio-Rad) was used to concentrate putative metal contaminants of various reagents. Chelex resin was washed in 50% HCl-water and then with water until no Cl^- was detectable by $AgNO_3$ reaction. One-tenth g of washed resin was mixed in a Beckman microfuge tube with 1 ml 100 mM rib 5P, $NaHCO_3$, $MgCl_2$, sorbitol, EDTA, or mercaptoethanol and left overnight. The resin was centrifuged from solution in a Beckman microfuge, the supernatant decanted, and the resin allowed to air dry for several hr. Then 15 μl of 50% HCl-water was mixed with the resin using a Vortex mixer. The resin was again centrifuged from solution, and the 10-μl supernatant recovered. The resin was washed with an

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[2] Abbreviations: RuDPCase (EC 4.1.1.39): ribulose bisphosphate carboxylase; rib 1,5diP: ribulose 1,5-diphosphate; 1,3diPGA: 1,3-diphosphoglyceric acid; rib 5P: ribose 5-phosphate; 3PGA: 3-phosphoglyceric acid.

additional 10 μl acid, and the two supernatants used as the sample for paper chromatography. Similar preparations from solutions containing known quantities of Pb^{2+} and Hg^{2+} indicated that the metal ion recovery in the acid fraction is about 90%. Metal ions were analyzed by paper chromatography on 20-cm square sheets of Whatman No. 1 chromatography paper in two solvent systems: 95% methanol-5% concentrated HCl or 87% methyl acetate-3% methanol-10% water (2). Metals were visualized as their dithizonate derivatives (ref. 3, reagent No. 93). This test is sensitive to 0.1 μg.

RESULTS AND DISCUSSION

In initial experiments with the complete assay mixture for RuDPCase the bizarre kinetic data shown in Figure 1 were obtained in which there is clearly an inverse relationship between rib 5P concentration and apparent reaction rate. The asymptotic approach of the curve to the $\frac{1}{v}$ axis at high substrate concentration suggests that a noncompetitive inhibitor of the reaction is present in the substrate. The minimum in the curve indicates the concentration of substrate at which too little inhibitor is present to affect reaction rate; at substrate concentrations below this the curve approaches a straight line from which the K_m and V_{max} of the reaction can be estimated as indicated in the figure legend.

Site and Source of Inhibition. To determine the source of inhibitor and the site of action, the effect of various potential inhibitors was measured on the partial reaction of 3PGA-dependent NADH oxidation (Table I). NADH oxidation is completely dependent on the addition of 3PGA, and the expected stoichiometry of 1 mol NADH oxidized/mol 3PGA added is observed in mixtures including the ATP regenerating system. No feedback inhibition by NAD^+ was observed since the reaction goes to completion with no diminution of rate. Addition of

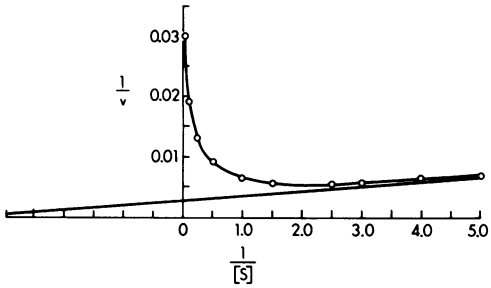

FIG. 1. Effect of varying ribose 5 phosphate concentration (1/(S) μmol) on the apparent rate of RuDPCase activity (1/v μmol/mg Chl/hr) measured spectrophotometrically (5). Complete reaction mixture contained 5 mM glutathione, 10 mM $MgCl_2$, 5 mM ATP, 1 mM NADH, 2.5 units glyceraldehyde 3-P dehydrogenase, 3.8 units 3PGA kinase, 1 unit creatine phosphokinase, 5 mM phosphocreatine, and 50 mM tris (pH 7.8) plus chloroplast extract equivalent to 5 μg Chl. This mixture was preincubated for 5 min, 20 C, prior to the addition of 0.5 μmol ribose-5-P. This sample of rib 5P contained 40 μg/g lead. Estimates of K_m, V_{max}, and K_i can be made by extrapolation of the asymptote: the y intercept is

$$\frac{1}{V_{max}}\left(1+\frac{xKm}{Ki}\right)$$

and the $-x$ intercept is

$$-\frac{\left(1+\frac{xKm}{Ki}\right)}{Km}$$

where x equals the proportion of contaminant in the substrate (7). Considering the multireactant nature of this assay, a more sophisticated kinetic analysis would be required to prove that metal contaminants have only a single inhibition site and action and hence only one K_i. With the assumption of a single K_i, x = 40 μg/g, V_{max} = 340 μmol/mg Chl·hr, and K_m = 20 μM (an assumption) the calculated K_i is 4 nM.

Table I. Effects of RuDPCase Reaction Mixture Components on the Rate of 3PGA Dependent NADH Oxidation

The complete reaction mixture contained 5 mM glutathione, 10 mM $MgCl_2$ 5 mM ATP, 1 mM NADH, 2.5 units glyceraldehyde 3-P dehydrogenase, 3.8 units 3PGA kinase, 1 unit creatine phosphokinase, 5 mM phosphocreatine, and 50 mM Tris pH 7.8. This mixture was preincubated for 5 min and then 0.2 mM 3PGA was added to make a final volume of 1.0 ml. The final reaction pH was 7.8, 20 C. Additions are indicated at final concentration in the reaction mixture. There was approximately a 5 sec delay between addition of 3PGA and initiation of recording. Data shown is average of 2 determinations. Complete mixture reaction rate: 180 nmol NADH oxidized/min.

Reaction Mixture	Lag Period	Initial Rate as % of Complete
	sec	
Complete	10	100
- 3PGA	∞	0
+ 5 mM phosphate, pH 7.5	10	89
+ 0.2 mM Rib 5P	35	49
+ 2.0 mM Rib 5P	125	11
+ 2.0 mM Rib 1,5diP	10	96
+ 2.0 mM Rib 5P at end of preincubation	10	First 30 sec 76 After 90 sec 35
+ 2.0 mM Rib 5P and 5 mM ATP	130	14

$NaHCO_3$, chloroplast extract, Pi, sorbitol, KCl, rib 1,5diP, or sulfate during preincubation had little effect on the reaction kinetics (data not shown). However, 2 μmol rib 5P added during preincubation suppress NADH oxidation to 10% of the control value and substantially increase the lag period; the extent of rib 5P inhibition is dependent both on the amount of rib 5P added and on the time of addition. Addition of rib 5P simultaneously with 3PGA after the preincubation period results in a biphasic reaction with a rapid initial rate (first 30 sec) followed by a period of progressively decreasing rate. No NADH oxidation is evident during preincubation with rib 5P. These results suggest that the inhibitory activity of the rib 5P preparation is due to a contaminant acting as a noncompetitive inhibitor of 3PGA kinase, glyceraldehyde-3-P dehydrogenase, or creatine phosphokinase.

The initial rate of 3PGA-dependent oxidation of NADH is reduced about 70% by the omission of the ATP-regenerating system, and the reaction rate is linear for only 1 min (data not shown). In the absence of the ATP-regenerating system, NADH oxidation is further reduced by addition of rib 5P. The per cent of control activity in the presence or absence of the ATP-regenerating system when rib 5P is added is equal, indicating that the inhibitory effect of rib 5P is preferential for the 3PGA kinase-glyceraldehyde-3-P dehydrogenase reactions and not for the ATP-regenerating system.

Likely contaminants of rib 5P were tested for inhibitory activity in the complete 3PGA-dependent reaction (Table II). Phosphate, other sugars, and free ribose had only a slight inhibitory effect at high concentration. Heavy metals, however, were potent inhibitors of the reaction at 100 nM concentrations. In the standard RuDPCase assay, 0.5 μmol of rib 5P is added; contamination of this reagent with 0.025 nmol heavy metal would account for the large inhibitory effects observed.

Preincubation of the reaction mixture with metal ions resulted in more inhibition than simultaneous addition of substrate and metal ions as was observed for the rib 5P inhibition. Treatment of metal solutions with Chelex-100, a chelating agent, prior to addition alleviated inhibition; even the addition of Chelex-100 to the reaction mixture followed by addition of metal solutions preserved some apparent enzyme activity. Chelex-100 has some adverse effects on the reaction probably as a result of its ability to remove Mg^{2+}. Treatment of rib 5P with Chelex-100 allowed the addition of 10 μmol rib 5P to the RuDPCase assay before any inhibitory activity was observed (Table III); this inhibitory effect is transient and reversible and increases the lag period only.

Thin layer chromatography of rib 5P indicated that over 99% of the material present was sugar phosphate and approximately 95% of the material was authentic rib 5P (data not shown). Other organic material present did not inhibit NADH oxidation. Paper chromatography of Chelex-100-treated reagents indicated that most reagents contain no trace contaminants of heavy metals of greater than 5 μg/g. Metal concentration was determined by comparison with a set of standards in the same chromatograph; standards of lead, mercury, zinc, manganese, silver, iron, and tin were used. Ribose-5-P was the only reagent with substantial heavy metal contamination; the major contaminant was lead which ranged from less than 5 to 100 μg/g in various lots of rib 5P. The experiments described in this study were done with a preparation that contained 40 μg/g lead. This quantity of lead in rib 5P is sufficient to account for the inhibition of NADH oxidation observed by the addition of rib 5P.

RuDPCase Assay Using Purified Ribose 5-Phosphate. Reexamination of the RuDPCase assay is shown in Table III. The effects of various additions are essentially identical to those found for the coupled system alone suggesting that the rib 5P inhibitor acts primarily on the labile kinase-dehydrogenase activity previously identified as the likely site of inhibition (data not shown). Ribose 5-phosphate purified by Chelex-100 treatment was free of inhibitory activity at low concentration. Reaction rate is dependent on substrate concentration and chloroplast concentration. The increase in lag period to 16 sec in the complete reaction mixture compared to 3PGA-dependent NADH oxidation probably reflects the requirement for rib 5P phosphorylation and isomerization by the chloroplast extract. In the complete reaction mixture, the linear rate of NADH oxidation is 680 μmol/mg Chl·hr which is equivalent to a rate of CO_2 fixation of 340 μmol/mg Chl·hr. At high rib 5P concentration, there is an increase in the lag period prior to initiation of measurable oxidation of NADH which proceeds at a linear rate approximately equal to the complete system after the lag period. The length of the lag period can be manipulated by varying the concentrations of rib 5P, ATP, and the ATP-regenerating system.

In the absence of the ATP-regenerating system, the lag period prior to measurable NADH oxidation is lengthened by low

Table II. Effects of Likely Contaminants of Ribose 5 Phosphate on 3PGA Dependent NADH Oxidation

Reaction mixture and conditions identical to Table I. Complete reaction rate: 182 nmol/NADH oxidized/min.

Reaction Mixture	Lag Period	Initial Rate as % of Complete
	sec	
Complete	10	100
100 nM $HgCl_2$	135	4
25 nM $HgCl_2$	25	24
100 nM $PbCl_2$	165	6
25 nM $PbCl_2$	40	31
100 nM $MnCl_2$	100	16
100 nM $ZnCl_2$	85	7
100 nM $FeCl_2$	65	38
1 ug Chelex-100	10	90
1 ug Chelex-100 + 0.01 uM $PbCl_2$	12	75

Table III. RuDPCase Assay with Purified Ribose 5-Phosphate

Complete reaction mixture contained 5 mM glutathione, 10 mM $MgCl_2$ 5 mM ATP, 1 mM NADH, 2.5 unites glyceraldehyde 3-P dehydrogenase, 3.8 units 3PGA kinase, 1 unit creatine phosphokinase, 5 mM phosphocreatine, and 50 mM Tris pH 7.8 plus chloroplast extract equivalent to 5 ug Chl. This mixture was preincubated for 5 min, 20 C, prior to the addition of 0.5 umole ribose 5P. Average of 2 determinations. Complete reaction rate: 680 u moles NADH oxidized/hr/mg Chl.

Reaction Mixture	Lag Period	Initial Rate as % of Control
	sec	%
Complete	16	100
- rib 5P	∞	0
- NADH	∞	0
- chloroplast extract	∞	0
+ 1.0 ug Chl	18	8
+ 4.0 ug Chl	16	85
+ 8.0 ug Chl	18	95
+ 0.1 mM rib 5P	16	80
+ 1 mM rib 5P	26	103
+ 5 mM rib 5P	47	100
+ 10 mM rib 5P	98	86
+ 20 mM rib 5P	180	80
+ 25 mM rib 5P	226	84
+ 25 mM rib 5P, 25 mM ATP	16	98

concentrations of rib 5P (data not shown). This effect is not due to metal contaminants because it is completely reversed by the addition of ATP or the ATP-regenerating system as would be expected from the observations of Slabas and Walker (9) on the necessity for maintenance of the appropriate ATP:ADP ratio for 3PGA kinase activity in reconstituted chloroplasts. The yeast NADH-dependent 3PGA kinase used in these experiments is also sensitive to ATP:ADP ratio (4).

In conclusion, it is clear that heavy metal impurities in substrates can have a major impact on RuDPCase apparent activity when assayed by this method. It is recommended that potential contaminants be removed by chelating resins or that an empirical test be performed to demonstrate that there are no noncompetitive inhibitors present in the assay mixture. To facilitate meaningful comparison of enzyme activity and kinetic properties, standard conditions including the exclusion of heavy metals must be met.

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