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Functional Importance of Arginine 64 in *Chlamydomonas reinhardtii* Phosphoribulokinase

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ABSTRACT

Phosphoribulokinase (EC 2.7.1.19) was investigated in wild-type *Chlamydomonas reinhardtii* and in mutant strains deficient in this enzyme activity. Immunoblot analysis revealed substantial amounts of phosphoribulokinase in mutant 12–2B but none in mutant F-60. The pH optimum of the wild-type enzyme was 8.0 and that of the 12–2B enzyme was 6.5. The mutant kinase possessed a K_m value for ribulose 5-phosphate of about 45 millimolar, nearly three orders of magnitude greater than the wild-type value of 56 micromolar. K_m values for ATP in the range of 36 to 72 micromolar were observed with both wild-type and mutant enzymes. The V_{max} of the wild-type enzyme was about 450 micromoles per minute per milligram of protein, and values for the mutant enzyme were 140 micromoles per minute per milligram at pH 6.5 and 36 micromoles per minute per milligram at pH 7.8. Thermal stabilities of the wild-type and mutant kinases were similar. Sequence analysis of the 12–2B phosphoribulokinase gene revealed a C to T transition that caused an arginine to cysteine change at position 64 of the enzyme. This arginine residue is conserved in phosphoribulokinases from vascular plants, algae, and photosynthetic bacteria and appears to function in binding ribulose 5-phosphate.

Phosphoribulokinase catalyzes the ATP-dependent phosphorylation of D-Ru5P[2] to form D-ribulose-1,5-bisphosphate, which is then a substrate for Rubisco in photosynthesis and photorespiration (18). Phosphoribulokinase plays a key regulatory role in the photosynthetic carbon reduction cycle, as evidenced by its multiple and diverse control mechanisms. The enzyme of eukaryotic organisms is regulated by metabolites (6), energy charge (12), and light via the Fd-thioredoxin regulatory system which reduces a specific disulfide to activate the enzyme (2, 4). Phosphoribulokinase from the green alga *Chlamydomonas reinhardtii* is similar in its structural, regulatory, and kinetic properties to the enzyme of higher plants and is encoded by a single-copy, nuclear gene (9, 26).

Few functional amino acid residues have been identified in phosphoribulokinase from any organism. No residues that participate in catalysis or in binding Ru5P have yet been clearly identified. Only a few residues of the ATP-binding domain have been identified through recognition of a nucleotide-binding consensus sequence (11, 21) and by affinity labeling the spinach enzyme with ATP analogs (11, 16). The reductive/oxidative regulation of phosphoribulokinase also is not clearly understood. Cysteines 16 and 55 are the regulatory cysteines for this thioredoxin-mediated regulation (15, 21), but the mechanism of inactivation associated with disulfide formation is not known.

Two *Chlamydomonas* mutant strains deficient in phosphoribulokinase activity have been isolated, 12–2B (27, 29) and F-60 (17). Both of these mendelian mutants require acetate for growth and have intact photosystems. Mutant 12–2B possesses substantial amounts of phosphoribulokinase protein, with an altered isoelectric point and measurable activity (27). Light activation of the 12–2B enzyme is not impaired. Little or no phosphoribulokinase activity was observed in extracts of mutant F-60 (16), but no additional characterization of the lesion has been done. Both mutations are expected to be point mutations, because the mutagens used were ethyl methanesulfonate for 12–2B and methyl methanesulfonate for F-60. The 12–2B and F-60 mutant strains were further examined in the present study.

MATERIALS AND METHODS

Strain and Culture Conditions

Chlamydomonas reinhardtii wild-type strain 2137 and mutant strain F-60 were grown mixotrophically at 27°C on acetate medium (29) at a light intensity of about 200 μmol photons m^{-2} s^{-1}. Because of its light-sensitive phenotype (29), mutant strain 12–2B was grown heterotrophically in the dark in the same medium. Air was continuously bubbled through the cultures.

Protein Purification and Immunoblot Analysis

Phosphoribulokinase from wild-type and mutant 12–2B strains was purified to apparent homogeneity as described previously (26) with two modifications. Gel filtration chromatography with a fast protein liquid chromatography Superose[3] 12 column was omitted, and affinity chromatog-

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[2] Abbreviations: Ru5P, ribulose 5-phosphate; kb, kilobase.

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raphy with ATP-agarose was performed twice rather than once. In the first ATP-agarose treatment with 20 mM bis-tris-propane and 5 mM DTT at pH 8.8, phosphoribulokinase did not bind but several impurities did. In the second treatment with 20 mM bis-tris-propane and 5 mM DTT at pH 7.0, phosphoribulokinase bound to the column and was eluted with ATP as reported previously (26). Protein was determined by the method of Bradford (1) with the Bio-Rad reagent, using BSA as a standard. Immunoblot analysis was done as described previously (26).

Assay of Phosphoribulokinase Activity

Enzyme activity was measured spectrophotometrically at 25°C by coupling Ru5P-dependent ADP formation to NADH oxidation with pyruvate kinase and lactate dehydrogenase (22). Buffers and Ru5P concentrations are reported in the figure legends. All other assay components and concentrations were as described previously (26). Substrates and enzymes for the assays were from Sigma.

Determination of Kinetic Parameters

Kinetic parameters were determined from double-reciprocal plots and linear regression analysis. V_{max} and K_m(Ru5P) values were determined with the data of Figure 3. K_m(ATP) values were determined at 2 mM Ru5P concentrations for the wild-type enzyme and 4 mM for the 12–2B enzyme.

Isolation and Sequencing of a Phosphoribulokinase Clone from Mutant 12–2B

Genomic DNA blot analysis of *Sma*I-digested 12–2B DNA revealed a 4-kb fragment that hybridized with a previously characterized (26) wild-type phosphoribulokinase cDNA. *Sma*I-digested 12–2B DNA was, therefore, size fractionated in a 0.8% low melting point agarose gel, and the DNA of approximately 3 to 5 kb was excised. A subgenomic library of this DNA in λgt11 was constructed using the procedure and materials from a Pharmacia LKB cDNA synthesis kit. Packaging extracts were from Promega Biotec. The library (about 135,000 plaques) was screened in *Escherichia coli* Y 1090 by hybridization with the wild-type cDNA, and six positive signals were identified. One of these recombinants contained an insert that was found to include 90% of the coding region for mature phosphoribulokinase, from within an intron between nucleotide positions 233 and 234 (numbering system from ref. 25) past the 3′ end of the coding region. This region was sequenced in entirety using a single-stranded template from M13 vectors and the dideoxy chain termination method, with the Sequenase kit (U.S. Biochemical) and α^{35}S-dATP. To complete the sequence, a second subgenomic library was constructed in pUC19 plasmid with *Bam*HI-digested mutant 12–2B genomic DNA. A 5.5-kb insert containing the phosphoribulokinase gene from the 5′ end to position 839 was isolated. A *Nco*I-*Hin*dIII fragment containing the coding region from position 26, which includes the ATG start codon, to within the intron between positions 233 and 234 was subcloned into plasmid M13 for sequencing as above. The mutant 12–2B-coding sequence was identical with wild type in this region.

RESULTS

Immunoblot Analysis

Immunoblots of crude protein extracts from wild-type, mutant F-60, and mutant 12–2B strains were probed with antibodies to the purified *Chlamydomonas* wild-type protein (Fig. 1). No phosphoribulokinase was detected in mutant F-60, even with five times more protein than wild type. A phosphoribulokinase polypeptide, similar in size to the wild-type polypeptide, was detected in mutant 12–2B. This result is consistent with a previous experiment in which an extract of 12–2B was probed with antibodies to the spinach protein (27).

pH Response

Chlamydomonas wild-type phosphoribulokinase displayed a broad activity peak in response to pH, with an optimum at pH 8.0 (Fig. 2). Similar results were previously reported for the spinach (12) and wheat (30) proteins. In contrast, the 12–2B enzyme displayed a narrower activity peak with an optimum at pH 6.5.

Determination of Kinetic Parameters

Results of the kinetic studies with purified wild-type and mutant 12–2B enzymes are summarized in Table I and Figure 3. The K_m(Ru5P) value for the mutant enzyme was approximately 45 mM, three orders of magnitude greater than the wild-type value of 56 μM. In contrast, K_m(ATP) values were similar for wild-type and mutant enzymes. V_{max} values for the 12–2B enzyme were lower than the wild-type values, but these reductions were relatively small compared to the corresponding changes in K_m values. Because of the possibility that the 12–2B enzyme had reduced substrate specificity, alternate substrates for Ru5P were tested. No activity was observed for the wild-type or 12–2B enzymes with 10 mM concentrations of xylulose-5-phosphate, fructose-6-phosphate or ribulose (not shown).

Thermal Stability

Thermal denaturation experiments were conducted with wild-type and mutant phosphoribulokinase to determine

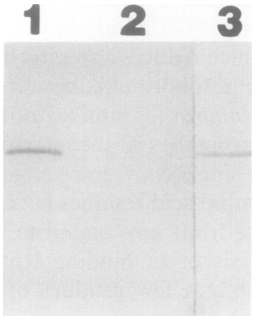

Figure 1. Immunoblots of *Chlamydomonas* crude protein extracts, probed with polyclonal antibodies to wild-type *Chlamydomonas* phosphoribulokinase. Lane 1, wild type (5 μg); lane 2, mutant F-60 (25 μg); lane 3, mutant 12–2B (5 μg).

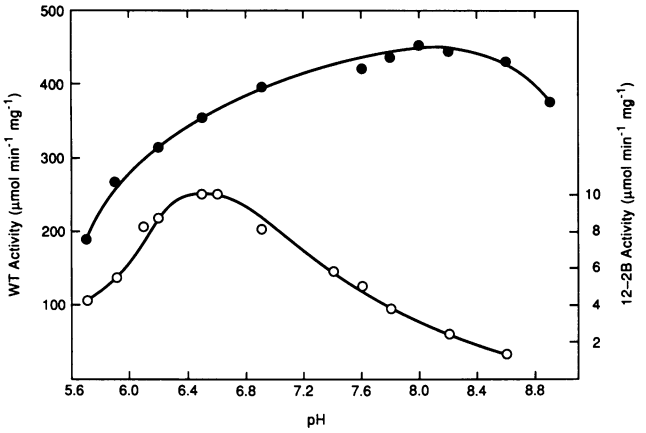

Figure 2. Effect of pH on activity of wild-type (WT, ●) and mutant 12–2B (○) phosphoribulokinase. Assays at pH 6.6 or less were run in 100 mM Mes-KOH. Assays at pH 6.9 or above were run in 50 mM Hepes, 50 mM Bicine-KOH. Ru5P concentrations were 0.5 mM for wild-type assays and 5 mM for mutant 12–2B assays.

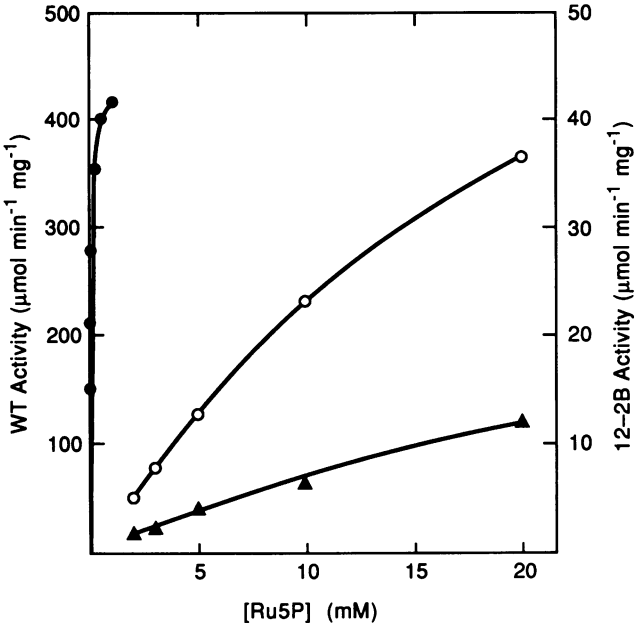

Figure 3. Ru5P dependence of wild-type and mutant 12–2B phosphoribulokinase activity. Wild-type (WT) assays (●) were run in 100 mM Tris-HCl, pH 7.8. 12–2B assays were run in 100 mM Mes-KOH, pH 6.5 (○), or in 100 mM Tris-HCl, pH 7.8 (▲). ATP concentration was 2 mM.

whether the 12–2B mutation substantially affected the three-dimensional conformation of the enzyme. No significant differences in the thermal stabilities of the wild-type and 12–2B kinases were observed (Fig. 4).

Sequence Analysis of the 12–2B Mutation

Sequence analysis of the 12–2B genomic clones and comparison with the wild-type cDNA sequence (26) revealed only one change, a C to T missense mutation that caused an arginine to cysteine change at position 64 of mature phosphoribulokinase (Fig. 5). This result is consistent with the previous observation that the 12–2B enzyme possesses a more acidic isoelectric point than does the wild-type enzyme (27). The primary structure of phosphoribulokinase has been determined for six organisms, representing higher plant monocotyledons and dicotyledons, green algae, and photosynthetic bacteria (Fig. 6). Arginine 64, corresponding to arginine 49 in bacterial phosphoribulokinase, is conserved in the enzyme from all of these species.

DISCUSSION

Arginine 64 in *Chlamydomonas* phosphoribulokinase is clearly not essential for catalysis, because substantial catalytic activity is retained in its absence in the 12–2B enzyme. However, there was a 1000-fold increase in the K_m(Ru5P) value. Retention of activity together with the large increase in K_m values in the 12–2B enzyme suggests that arginine 64 functions in binding Ru5P. An additional role in facilitating catalysis also cannot be ruled out, because decreases in V_{max}, although relatively small, were observed with the 12–2B enzyme. The mutation appeared to cause no large changes in conformation, because the thermal stabilities of the wild-type and mutant enzymes were similar. The mutation did not prevent assembly into a dimeric holoenzyme, because identical elution profiles for wild-type and 12–2B enzymes were observed during gel filtration chromatography with Sephacryl S-300 (performed as in ref. 26). The arginine to cysteine change results in a smaller side chain at position 64 of the 12–2B enzyme, making steric hindrance an unlikely explanation for the increased K_m value of the mutant kinase. Substantial precedent exists with numerous enzymes for a role of arginine residues in binding sugar-phosphate substrates, often through interactions with the phosphate group (3, 24). An indirect role for arginine 64 in binding Ru5P, through stabilization of a conformation conducive to binding, is also possible.

The importance of arginine 64 for proper function of phosphoribulokinase is further indicated by the fact that it is conserved in the enzyme from diverse organisms (Fig. 6). The presence of this residue in bacterial phosphoribulokinase is especially noteworthy, because the bacterial enzyme possesses <15% sequence identity (10) in comparison with the *Chlamydomonas* and higher plant enzymes (14, 23, 25, 26). The conserved arginine is located in one of five sequence motifs present in phosphoribulokinase of both prokaryotes and eukaryotes (10).

Phosphoribulokinase cysteines 16 and 55 form a disulfide

Table I. *Kinetic Parameters of Wild-type and Mutant 12-2B Phosphoribulokinase*

	Wild Type pH 7.8	12-2B	
		pH 7.8	pH 6.5
K_m(Ru5P), μM	56	42,000	49,000
K_m(ATP), μM	63	72	36
V_{max}, μmol min^{-1} mg^{-1}	450	36	140

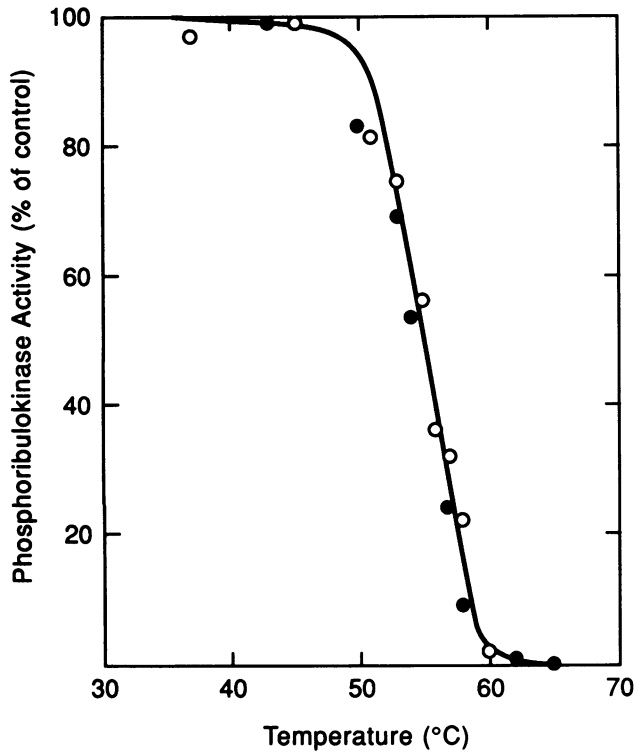

Figure 4. Thermal stability of wild-type and mutant 12–2B phosphoribulokinase. Aliquots of protein in 50 mM Hepes-KOH and 10 mM $MgCl_2$ (pH 7.5) were incubated at the indicated temperatures for 10 min before being returned to 4°C and subsequently assayed for activity. Control values, representing aliquots kept at 4°C at all times, were 410 μmol min^{-1} mg^{-1} for wild type and 15 μmol min^{-1} mg^{-1} for 12–2B. Wild-type assays (●) were run in 100 mM Tris-HCl, pH 7.8, and 0.5 mM Ru5P. 12–2B assays (○) were run in 100 mM Mes-KOH (pH 6.5), 20 mM ribose-5-phosphate, and 10 units phosphoriboisomerase.

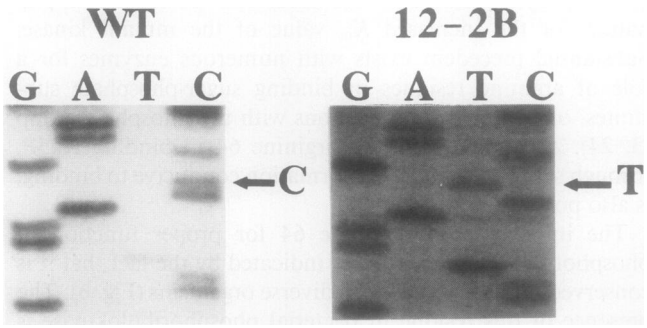

Figure 5. Sequence analysis of the 12–2B mutation. Arginine (CGC) was changed to a cysteine (TGC) in the 12–2B mutant. WT, Wild type.

C. reinhardtii (12-2B)	53-69	V I C L D D Y H C L D **C** N G R K V
C. reinhardtii (WT)	53-69	V I C L D D Y H C L D R N G R K V
Spinach	53-69	V I C L D D F H S L D R N G R K V
Wheat	53-69	V I C L D D Y H S L D R T G R K E
A. eutrophus	38-54	V I E G D S F H R Y D R A E M K V
R. sphaeroides	38-54	S I E G D A F H R F N R A D R K A
X. flavus	38-54	F V E G D S F H R Y D R Y E M R E

Figure 6. Comparison of phosphoribulokinase amino acid sequences. The asterisk indicates the arginine residue that was replaced with cysteine in *Chlamydomonas* mutant 12–2B. Sequences are from references 7, 10, 13, 14, 23, 25, and 26. WT, Wild type.

bond during oxidative inactivation (21). Loss of the cysteine 55 sulfhydryl accounts for much of the inactivation, because replacement of this residue with serine or alanine reduces activity by 85 to 90% (15). The arginine to cysteine change at position 64 of the 12–2B enzyme did not prevent this oxidative-reductive regulation. The wild-type and 12–2B enzyme exhibited the same degree of activation by light (21). Furthermore, incubation of both purified enzymes with 20 mM DTT restored activity lost during long-term storage (data not shown).

Arginine 64 may be in close proximity to cysteine 55 in the tertiary, as well as the primary, structure. Cysteine 55 of the spinach enzyme was labeled, and the enzyme was inactivated with the Ru5P analog bromoacetylethanolamine phosphate (20). Cysteine 55 was also cyanylated and the enzyme was inhibited with 2-nitro-5-thiocyanobenzoate (19). Ru5P provided protection against inactivation in both cases. However, analysis of these experiments was complicated by the fact that ATP also protected against inactivation.

ATP analogs have also been used to label the spinach enzyme. Lysine 68 was labeled and the enzyme was inactivated with adenosine triphosphopyridoxal, and ATP provided protection against inactivation (16). This residue corresponds to lysine 53 of the bacterial enzyme, but it is not completely conserved (Fig. 6), and its function is not known. Ultimately, a clear understanding of the position of arginine 64 in relation to lysine 68 or cysteine 55 may require solution of the crystal structure of phosphoribulokinase.

The cysteine substitution for arginine at position 64 resulted in a markedly different pH profile (Fig. 2). This may reflect ionization of the sulfhydryl group of the cysteine 64 side chain in the 12–2B enzyme at high pH, resulting in a perturbed active site environment. A definitive explanation for the different pH response is not possible at present, however.

The 12–2B strain grows on acetate as a carbon source but does not grow photoautotrophically (29), even though the mutant phosphoribulokinase possesses considerable activity. The concentration of stromal pentose monophosphates *in vivo*, including Ru5P, is only 0.3 to 0.4 mM, based on 9.3 nmol/mg Chl (5) and assuming 25 μL of stromal volume/mg Chl (28). This concentration of total pentose monophosphates is far below the K_m(Ru5P) value observed with the 12–2B enzyme. Concentrations of Ru5P are probably greater in the

mutant, because more accumulation of ^{14}C label in pentose plus hexose monophosphates was observed in the mutant strain than in wild type (27). However, the Ru5P concentration in the mutant strain apparently does not increase sufficiently to permit photoautotrophic growth. The pH of the chloroplast stroma in the light is about 8.0 (31), which is well above the pH optimum of the mutant enzyme (Fig. 2) and would further reduce phosphoribulokinase activity.

The *Chlamydomonas* F-60 mutant was isolated and characterized as deficient in phosphoribulokinase activity more than 20 years ago (17). This mutant strain has been used in various experiments including fermentative metabolism and CO_2 uptake studies (8). Despite this experimental use, it was not known whether the deficiency in activity reflected a catalytically defective enzyme or subnormal amounts of the enzyme. The immunoblot analysis presented here indicates that the phosphoribulokinase protein does not accumulate to detectable levels in this mutant strain. The precise nature of the F-60 lesion has yet to be determined.

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