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Final ONR Report

INTRODUCTION

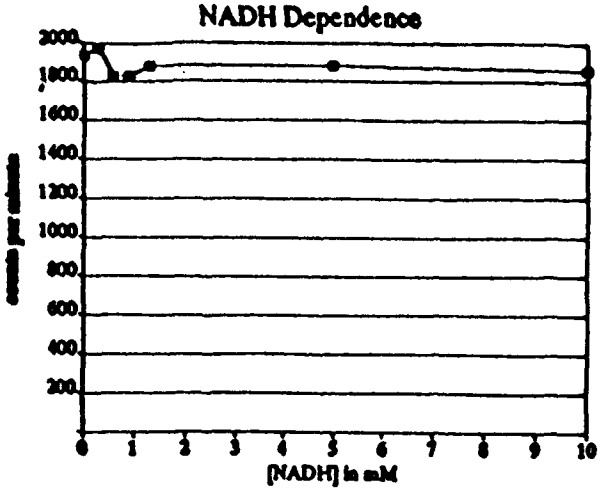
Global CO_2 (a "greenhouse" gas) levels have increased significantly in the last century. This increase has been greatly impacted by anthropomorphic activity, especially the burning of fossil fuels. Geochemists have argued that elevated CO_2 concentration in the atmosphere will eventually result in a detrimental change in climatic conditions earth-wide.

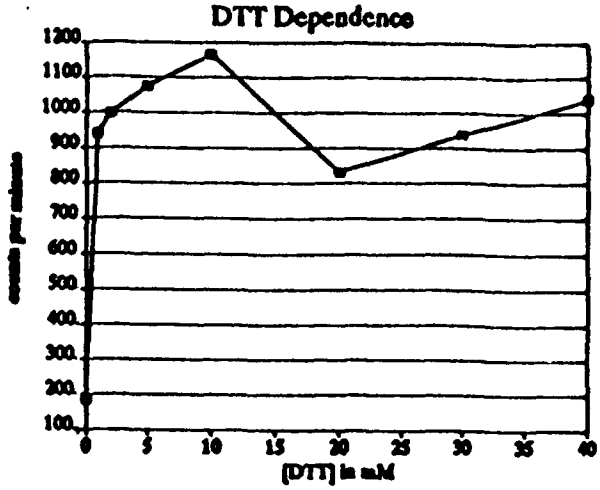
The process by which all plants and some bacteria convert CO_2 into more complex organic molecules is known as autotrophy. Plants and bacterial cells utilized either photosynthesis or chemosynthesis as an energy source to drive this process. Until recently, molecular information on the autotrophic process in eukaryotes has been derived almost exclusively from *terrestrial* plants and green algae (the Chlorophyta). However, two additional supertaxa of plants also fix CO_2. The Chromophyta represent a large assemblage of algae that include the diatoms and dinoflagellates, the most prevalent plankton in the ocean. Also included in this taxon are macrophytes that form large underwater forests. The rhodophytes, like the chromophytes, encompass unicellular as well as highly differentiated multicellular forms. These algal primary producers are also critical to the maintenance of marine ecosystems especially in anarctic waters. Both chromophytes and rhodophytes contain pigment arrays (e.g. fucoxanthin, peridinin, phycobili proteins) that allow them to utilize unique portions of the light spectrum for photosynthesis that are unavailable to chlorophytes. Questions concerning algal function continue to have importance. The presence of marine plants definitely effects the global carbon equation - approximately *half* of the total CO_2 fixed world-wide is processed by marine algae.

Presently, three mechanisms have been described by which CO_2 may be autotrophically processed. The reductive citric acid cycle and acetyl CoA condensation reaction are found exclusively in the green, methanogenic, and acetogenic bacteria (Schafer et al., 1989; Hemming and Blotevogel, 1985; Fuchs, 1986; Fuchs and Stupperich, 1985). In contrast, the Calvin cycle (McFadden and Tabita, 1974) is utilized by bacteria, cyanobacteria, and all eukaryotic plants. Thus this method of CO_2 processing represents the most universal method of fixation that is used among autotrophs.

Although it is accepted that the Calvin cycle is ancient in an evolutionary sense (carbon isotope fractionation suggests over 3.5 billion years), the origin of this cycle is not at all understood. Two enzymes, Ribulose 1,5 bisphosphate carboxylase (RUBP) and phosphoribulose kinase (PRK) are unique to Calvin cycle function and probably provided the evolutionary "break through" with respect to the biogenesis of this metabolic pathway, since the remaining Calvin cycle enzymes have additional (i.e. non-Calvin cycle) metabolic responsibilities.

There is no question that Rubisco has received much attention by investigators (e.g. Newman and Cattolico, 1990; Glover, 1989; Meager et al, 1989; Andrews and Lorimer, 1987; Tabita, 1988 for review) in both prokaryotes and eukaryotes (including marine algal representatives). In contrast, no information concerning the biochemistry or molecular biology of PRK exists for *any* marine plant.





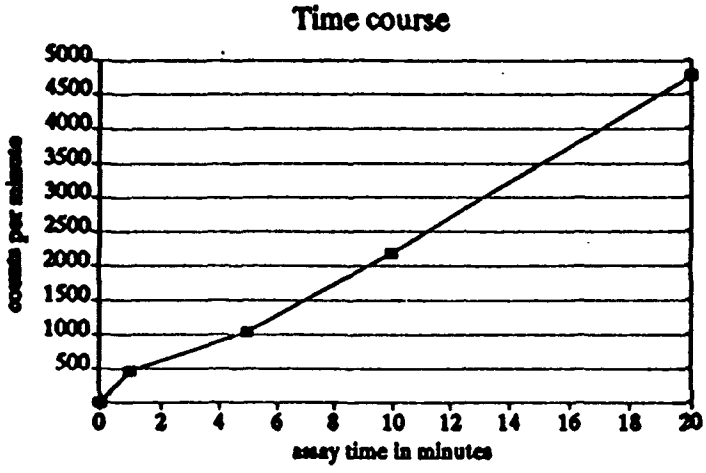


FIG. 1: Parameters of *Heterosigma* PRK activity (A) NADH dependence, (B) DTT dependence, (C) Time course.

TABLE 1: Heat Stability of *Heterosigma* PRK

Enzyme Treatment	Unboiled Control	Boiled	Remaining Activity (%)
Tris, DTT (1.0 mM)	12,207	7,361	60
Tris, DTT (20 mM)	12,303	6,496	53
Tris, DTT (20 mM), ATP	13,915	2,844	20
Tris, DTT (20 mM), $MgCl_2$	11,605	949	8
Tris, DTT (20 mM), ATP, $MgCl_2$	11,620	246	<1

Enzyme was boiled 5 min in the presence of reagents listed under enzyme treatment. Missing assay regents were then added before PRK activity levels were determined by standard assay for 20 min. Units in counts per minute.

WORK ACCOMPLISHED

The following data summarizes the work we have done on *Heterosigma* PRK isolation and characterization.

I. PRK PROTEIN CHEMISTRY

(A) Chloroplast isolation: Since PRK functions in the chloroplast isolation of this organelle immediately effects a large purification step. *Heterosigma* cells are harvested at L6 in the synchronous cell cycle when neither chloroplast replication, DNA synthesis, nor cell division is taking place. Approximately 4 liters of cells are gently centrifuged and then resuspended in chloroplast isolation buffer (Reith, 1985). The wall-less nature of *Heterosigma* causes this organism to be highly fragile, and gentle shear force using a narrow bore pipette can therefore be successfully used to break the cells. Chloroplast release is monitored by fluorescence microscopy. Plastids retrieved by differential centrifugation are resuspended in 100 mM Tris, 1 M mDTT buffer (pH 8.0) and frozen at -20°C.

(B) Enzyme purification: Both freeze/thawing and passage of *Heterosigma* chloroplasts through a 30 gauge needle helps to disrupt plastid membranes. Dispersed plastids are spun at 3,000 for 15 min and the supernatant layered over a 5-9-15% sucrose step gradient. Fractions are monitored for PRK activity using the following two step assay:

$$5\ \text{Ru P} \xrightarrow[\text{ATP}\ \curvearrowright\ \text{ADP}]{\text{PRK}} 1,5\ \text{Ru P} \xrightarrow[{}^{14}\text{CO}_2]{\text{Rubisco}} 3\ \text{PGA} - {}^{14}\text{C}$$

in which $^{14}CO_2$ incorporation into phosphoglyceric acid is measured (Marsden and Codd, 1984). Fractions containing PRK are pooled and concentrated to approximately 500 uL using a Centricon 30 (m wt. cut off 30 kda). Glycerol is added to 20% and this partially purified PRK preparation is frozen in aliquots for future use.

(C) Enzyme characterization: Sucrose gradient purification show that *Heterosigma* PRK neither exists in a large Calvin cycle enzyme complex as observed in terrestrial plants (Gontero et al., 1988; Sainis and Harris, 1986), nor appears to have a latent, large molecular weight enzyme that is seen in the fresh water alga *Scenedesmus*. *Heterosigma* PRK is well separated from its Rubisco (mwt 550,000) by this gradient purification step (data not shown). *Heterosigma* PRK activity is not dependent upon NADH but does require DTT (Fig. 1a, b), ATP and 5 Ru P 5 P appears to be stimulated by the presence of PEP (data not shown). The reaction is linear with respect to the amount of enzyme extract added (data not shown) and linear with time (Fig. 1c). Under the conditions used in this assay (see proposed research for discussion) the enzyme displays pH and temperature optima of 8.5 and 45°C respectively. As seen in Table 1, the *Heterosigma* PRK appears to be quite heat stable. Enzyme in the presence of DTT retained approximately 50% of its activity after 5 min of boiling. In contrast, boiling the enzyme in the presence of ATP or Mg++ appears to eliminate sensitivity to boiling.

Significance: Data show that a partially purified PRK has been recovered from the alga, *Heterosigma carterae*. These data verify that the heat stable enzyme is dependent on ATP, 5 Ru P and DTT for activity. NADH does not appear to influence enzyme activity as seen in purple

bacteria. The enzyme does not exist in a large multienzyme complex as documented for both pea and *Scenerdesmus* when PRK is isolated from cells harvested at L6 in the diel cycle.

II. GENETIC INFORMATION

The PRK coding site in *Heterosigma* is presently unknown. We have attempted to use both the *Rhodobacter sphaeroides* as well as the *Chlamydomonas* and Spinach PRK genes to probe both nuclear and chloroplast *Heterosigma* DNA with negative results. The low stringency needed for such heterologous analysis produced false positives which cost much sequencing effort. *Heterosigma* has not only a B purple-like Rubisco, but a purple bacterial -like orientation of its chloroplast encoded Rubisco operon (see Introduction). The purple bacteria *Rhodobacter sphaeroides* (Gibson et al., 1990) and *Alcaligenes eutrophus* (Windhövel and Bowien, 1990) encode PRK and Rubisco within the same operon. Extensive sequencing of the *Heterosigma* chloroplast genome in the vicinity of the Rubisco operon has given negative results. Thus far, attempts to retrieve the PRK gene with highly degenerate PCR primers made for either purple or Chlorophytic plant PRK's were also unsuccessful.

Significance: Although the preliminary biochemical data suggests that the *Heterosigma* Rubisco may have a terrestrial plant-like function, there appears to be little similarity at the DNA level for this gene to either purple bacterial or chlorophytic plant hybridization probes. Extensive and careful analysis have failed to identify PRK and its coding location in *Heterosigma*, suggesting that an additional evolutionary lineage(s) may exist for this enzyme.

SIGNIFICANCE

Although this study did not result in immediate publishable information, it has established a data base that will serve as the nucleus for further studies. These include:

(A) Continued Studies on *Heterosigma* PRK. A recently awarded NSF grant request will allow continuation of this study. The goals of the NSF request are: (1) isolate and purify PRK to homogeneity. (2) Generate a hybridization probe: micro sequencing the protein should allow construction of appropriate oligonucleotide probes which will be used to locate the coding site of the PRK gene which will be sequenced, phylogenies constructed, and putative functional domains assessed. (3) Analyze the effect of environmental cues on PRK: antibodies generated to the protein will be used in conjunction with appropriate PRK sequences to analyze the effect light has on PRK protein and gene regulation.

(B) Analysis of an Origin of Replication. False PRK signals led us to sequence over a 4 kb region of *Heterosigma* cpDNA. Through these studies we located a putative chloroplast DNA origin. Studies in collaboration with Genetics (U. of Washington) are in progress to define the function of this DNA domain.

(C) Contribution to Student Education. Few laboratories exist in the United States wherein molecular techniques established for terrestrial plant chloroplast gene function are applied to marine plants. This study served as a nucleus for the introduction of contemporary techniques to the chromophytic algae. These autotrophic plants have received little attention with respect to genome characterization and protein function.

Special note: I am grateful for this support which allowed us to pursue totally untouched questions concerning Calvin cycle function.

The persons supported by this grant have remained in the technical work force. For example, Ann Reynolds (Post-doctoral fellow) is presently constructing educational genetic films and Carrine Blank, after working as a technician, will begin her Ph.D. graduate education this fall.

REFERENCES

Andrews, T.J. and Lorimer, G.H. (1987) Rubisco: Structure, mechanisms, and prospects for improvement. In: The Biochemistry of Plants, P.K. Stumpf, E.E. Conn (eds.) Vol. 10. Academic Press, London, pp. 131-219.

Fuchs, G. (1986) CO_2 fixation in acetogenic bacteria: variations on a theme. FEMS Microbiol. Rev. 39:181-213.

Fuchs, G. and Stupperich, E. (1986) Carbon assimilation pathways in archaebacteria. System. Appl. Microbiol. 7:364-369. Gibbs, S.P. 1981. The chloroplasts of some algal groups may evolved from endosymbiotic eukaryotic algae. Ann. N.Y. Acad. Sci. 361:193-207.

Gibson, J.L., Chen, J-H., Tower, P.A., and Tabita, F.R. (1990) The form II fructose 1,6-bisphosphatase and phosphoribulokinase genes form part of a large operon in *Rhodobacter sphaeroides*: Primary structure and insertional mutagenesis analysis. Biochem. 29:8085-8093.

Glover, H.E. (1989) Ribulosebisphosphate carboxylase/oxygenase in marine organisms. Int. Rev. Cytol. 115:67-138.

Gontero, B., Cárdenas, M.L., and Ricard, J. (1988) A functional five-enzyme complex of chloroplasts involved in the Calvin cycle. Eur. J. Biochem. 173:437-443.

Hemming, A. and Blotevogel, K.H. (1985) A new pathway for CO_2 fixation in methanogenic bacteria. TIBS 198-200.

McFadden, B.A. and Tabita, F.R. (1974) D-ribulose-1,5-diphosphate carboxylase and the evolution of autotrophy. BioSys. 6:93-112.

Marsden, W.J.N. and Codd, G.A. (1984) Purification and molecular and catalytic properties of phosphoribulokinase from the cyanobacterium *Chlorogloeopsis fritschii*. J. Gen. Microbiol. 130:999-1006.

Meagher, R.B., Berry-Lowe, S., and Rice, K. (1989) Molecular evolution of the small subunit of ribulose bisphosphate carboxylase: nucleotide substitution and gene conversion. Genetics 123:845-863.

Newman, S.M. and Cattolico, R.A. (1990) Ribulose bisphosphate carboxylase in algae: synthesis, enzymology and evolution. Photosyn. Res. 26:69-85.

Reith, M. and Cattolico, R.A. (1985) *In vivo* chloroplast protein synthesis by the chromophytic alga *Olisthodiscus luteus*. Biochem. 24:2556-2561

Sainis, J.K. and Harris, G.C. (1986) The association of ribulose-1,5-bisphosphate carboxylase with phosphoriboisomerase and phosphoribulokinase. Biochem. Biophys. Res. Comm. 139(3):947-954.

Schäfer, S., Götz, M., Eisenreich, W., Bacher, A., and Fuchs, G. (1989) ^{13}C-NMR study of autotrophic CO_2 fixation in *Thermoproteus neutrophilus*. Eur. J. Biochem. 184:151-156.

Tabita, F.R. (1988) Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganism. Microbiol. Rev. 52:155-189.

Windhövel, U. and Bowen, B. (1990) On the operon structure of the *cfx* gene clusters in *Alcaligenes eutrophus*. Arch. Microbiol. 154:85-91.