The Ribulose Bisphosphate Carboxylase/Oxygenase of *Prochlorothrix hollandica:* Purification, Subunit Structure and Partial N-Terminal Sequence Analysis of the Large Subunit

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Dedicated to Professor Wilhelm Menke on the occasion of his 80th birthday

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Ribulose bisphosphate carboxylase/oxygenase was purified to apparent homogeneity from the carboxysomes of *Prochlorothrix hollandica.* The MW of the native enzyme was estimated to be 560,000 Dalton, comprising large subunits (LSU) of 57,000 Dalton and small subunits (SSU) of 13,000, probably in an 8LSU8SSU quaternary structure. Enzyme activity was maximal at pH 8.0 at 30 °C. The requirement of activity for Mg^{2+} could not be replaced by Mn^{2+}, Co^{2+}, Ca^{2+} or Cu^{2+}. Amino acid N-terminal sequence analysis of the LSU showed a high degree of conservation when compared to cyanobacterial and chloroplast LSU sequences but was too short to allow a reliable phylogenetic assignment of *P. hollandica.*

Introduction

The Prochlorophyta (also termed Prochlorales in the class Photobacteria) are a group of oxygen-evolving photosynthetic prokaryotes containing chlorophylls *a* and *b* but lacking phycobilins [1–7]. Their possession of chlorophyll *b* and lack of phycobilins provided an early indication that the prochlorophytes were distinct from the cyanobacteria [7–9] and their pigment complement is typical of the chloroplasts of green algae and higher plants. However, prochlorophytes are typically prokaryotic in terms of ultrastructure, genetic organization and several biochemical characteristics [7–9].

Until recently, the symbiotic species of the genus *Prochloron* were the only known members of the group and although the biological and evolutionary significance of this class of organism was recognized, investigations have been constrained by the inability to culture *Prochloron* independently of it's ascidian host species. Since the exciting discovery of the free-living, filamentous *Prochlorothrix hollandica* from a shallow eutrophic freshwater lake in the Netherlands [5] and a novel free-living, marine unicellular prochlorophyte abundant in the oceanic euphotic zone [10], opportunities have increased to study the biochemical characteristics and evolutionary position of prochlorophytes in the consideration of an ancestral origin of higher plant chloroplasts. This prospect has been the focus of much concern recently, although no clear conclusions have yet been reached [11, 12].

In this paper we report on the purification, structure and properties of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from *P. hollandica.* Previous studies of this major CO_2-fixing enzyme from various prokaryotic and eukaryotic sources have led to hypotheses concerning the molecular evolution of autotrophy [13–16]. Prochlorophytes have been shown to contain RuBisCO [17–19] consistent with their ability to assimilate CO_2 *via* the Calvin cycle [20]. Some of the RuBisCO is sequestered in carboxysomes [18, 19, 21, 23]. RuBisCO has been partially purified from *Prochloron,* the symbiont of *Lissoclinum patella* using freeze-dried field material [17]. Here we report on the purification of RuBisCO from *P. hollandica* grown in the laboratory in mass culture and on the N-terminal amino acid sequence of the large subunit (LSU) of RuBisCO. The purpose of the study was to assess the degree of physical, structural and molecular homology of RuBisCO from the free-living with that of the symbiotic prochlorophyte and RuBisCO's from cyanobacteria, green algae and higher plants.

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Materials and Methods

Organism and growth conditions

Prochlorothrix hollandica was a gift from Dr. T. Burger-Wiersma and Prof. L. R. Mur, University of Amsterdam and was grown in FPG medium as detailed elsewhere [6]. Cells were grown photoautotrophically in 20 l batch cultures, continuous illumination being provided at a photon fluence rate incidence on the vessels of 300 μ $Em^{-2} \cdot s^{-1}$.

Preparation of cell-free extracts and enzyme purification

5–8 g wet weight of cells were harvested from 15 l of culture by continuous centrifugation. All subsequent steps were performed at 4 °C unless otherwise stated. Cells were resuspended in low Tris isolation buffer, pH 8.0 (LTIB; 10 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA · Na_2, 50 mM $NaHCO_3$, 12 mM mercaptoethanol). Cells were broken by passage through a French pressure cell (110 MPa). Remaining whole cells and large membrane fragments were removed by low-speed centrifugation. The cell-free extract was centrifuged at 40,000 × *g* × 1 h and the pellet was then used as the starting material for further purification.

The 40,000 × *g* pellet was resuspended in 3–4 ml of LTIB and solubilized by ultrasonication using an MSE 9.5 mm probe at full power for 3 × 30 s bursts with intermittent cooling. The solubilized pellet was then centrifuged again at 40,000 × *g* × 1 h. The pellet was discarded and the supernatant was further centrifuged at 100,000 × *g* × 1 h. Again the pellet was discarded, solid $(NH_4)_2SO_4$ was then added to the brown supernatant to 30% saturation followed by centrifugation at 15,000 × *g* × 15 min. The supernatant was adjusted to 50% $(NH_4)_2SO_4$ saturation which resulted in precipitation of the bulk of the high molecular weight proteins. The pellet was resuspended in about 2.0 ml LTIB and dialyzed against 2 l of the same buffer overnight. 1–1.5 ml of the dialyzed enzyme solution was layered onto a 17 ml linear sucrose density gradient (0.2–0.8 M in LTIB) and centrifuged at 240,000 × *g* × 80 min in a TV 865 vertical rotor in a Sorvall OTD 65B ultracentrifuge). 0.5 ml fractions were collected which were retained for enzyme assay, protein determination and refractive index measurements.

Enzyme assay

RuBisCO activity was measured as RuBP-dependent incorporation of $NaH^{14}CO_3$ into acid-stable material as described elsewhere [21]. Acid-stable radioactivity was determined in a Packard Minaxi Tri Carb 4000 counter using the external standard ratio method.

Protein determination

Protein was measured using both the micro-Lowry [24] and Bradford methods [25].

Molecular weight determination

The MW of the native enzyme was determined by gel filtration using Sephacryl S-300 (Pharmacia) in a 1.5 m column calibrated with standards. The column was eluted with 50 mM Tris-C' pH 8.0. The MW of the subunits were estima by comparing their electrophoretic mobilities a SDS-PAGE with appropriate standards [22].

Large subunit amino-terminal sequence analysis

Samples of purified enzyme (100–150 μg) were loaded into 15% (w/v) linear SDS-polyacrylamide gels containing 4 M urea using the buffer system of Laemmli [26]. After electrophoresis, gels were soaked in Tris-glycine buffer (25 mM Tris, 200 mM glycine) for 30 min. Proteins were electroblotted [27] onto polyvinylidene difluoride membrane (Immobilon transfer, 0.6 μm pore) at 0.1 A in the same buffer for 12 h. The membrane was rinsed in deionized water, stained with 0.1% (w/v) Coomassie blue R-250 in 50% (v/v) methanol for 5 min and destained for 30 min in 10% (v/v) acetic acid, 50% (v/v) methanol. The membrane was then rinsed in deionized water, air-dried and stored at −20 °C.

A small amount of the LSU had been proteolyzed from 57 kDa to 52 kDa. A strip was cut from the membrane corresponding to the 57 kDa band. Sequencing was carried out on the automated gas-phase sequenator at the University of Cambridge, Department of Biochemistry Protein Sequencing Facility.

Results

Purification of RuBisCO

RuBisCO was present at approximately equal specific activity and abundance in the initial 40,000 × *g* × 1 h supernatant and pellet fractions of cell-free extract. These fractions were derived from the cytoplasmic and particulate (carboxysome-containing) pools of the cell [21, 22]. Initial attempts were made to purify *P. hollandica* cytoplasmic RuBisCO. Usually RuBisCO's from autotrophic prokaryotes and chloroplasts can be readily separated from the majority of the soluble proteins of the respective cytoplasm or stroma by centrifugation of the 30–50% $(NH_4)_2SO_4$-precipitated and dialyzed material through sucrose density gradients [15, 22]. When this procedure was used with *P. hollandica* cytoplasmic extract, one major protein band with high RuBisCO activity was obtained in sucrose gradients. Polyacrylamide gel electrophoresis (PAGE) of peak RuBisCO-containing fractions from the sucrose gradient revealed one major band (RuBisCO) plus at least 6 contaminating proteins. Passage of peak cytoplasmic RuBisCO fractions through a second sucrose density gradient followed by several variations in fast performance liquid chromatography (FPLC, Pharmacia) did not separate a major contaminating protein from the RuBisCO (unpublished observations) and attempts at purification were concentrated on the *P. hollandica* carboxysome-containing particulate fraction. This fraction gave a major protein band in the centre of the sucrose gradient (Fig. 1) without the predominant accumulation of proteins towards the top of the gradient which is characteristic of RuBisCO purifications from cytoplasmic protein sources [21, 22]. The distribution of protein in the centre of the sucrose gradient essentially coincided with that of RuBisCO activity (Fig. 1). PAGE of the peak

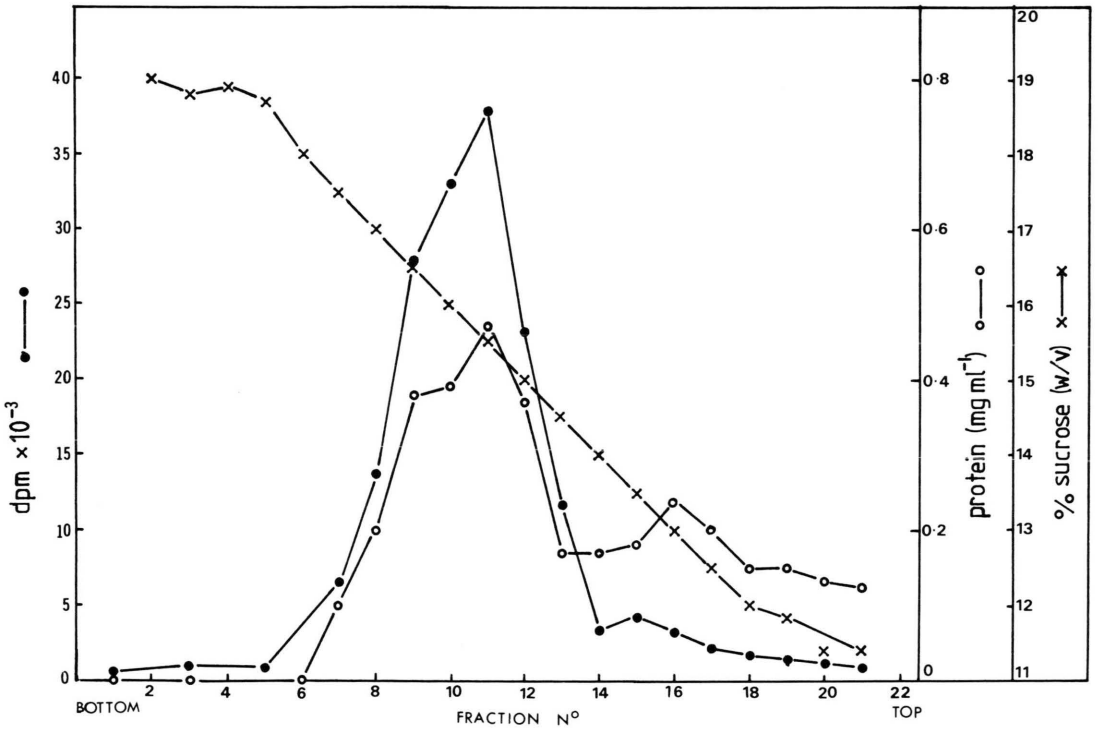

Fig. 1. Sucrose density gradient centrifugation of a 30–50% $(NH_4)_2SO_4$ fraction of solubilized particulate (carboxysome-containing) extract of *Prochlorothrix hollandica*. RuBisCO activity (RuBP-dependent incorporation of ^{14}C into acid-stable material) is expressed as dpm incorporated per 2 min assay [22]. Protein distribution was monitored as absorbance at 280 nm and sucrose by refractometry. Gradient fractions are numbered in the order in which they were withdrawn from the bottom of the tube.

RuBisCO activity fractions gave a single protein band (Fig. 2a). It was therefore decided to use the particulate cell-free extract as starting material for further purifications.

The enzyme was purified approximately 117-fold with a high specific activity of 0.8 μmol CO_2-fixed $min^{-1} \cdot mg\ protein^{-1}$. The final activity yield from the total broken cell extract was only 8.0% but this reflects that only the particulate fraction of the total complement of RuBisCO was purified. The amount of particulate *i.e.* carboxysomal RuBisCO varies depending on growth conditions and it is likely that as in cyanobacteria, particulate and soluble RuBisCO are structurally, biochemically and immunologically identical enzymes [22, 23]. The enzyme was estimated to be 92% pure as determined by densitometric scanning using a Joyce Loebl Chromoscan 3.

Molecular weight and subunit structure

The enzyme was found to consist to two subunit classes as determined by SDS-PAGE. The MW of the large subunit (LSU) and small subunit (SSU) were 57 kDa and 13 kDa respectively (Fig. 2b). Assuming a typical prokaryotic 8L8S structure, the native enzyme would have had a MW of 560 kDa. This value was identical with that obtained by gel filtration of a semi-purified enzyme preparation [21].

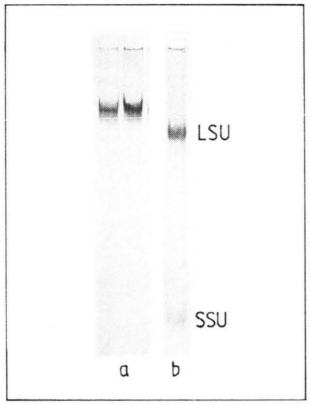

Fig. 2. (a) Standard PAGE of RuBisCO purified from the carboxysomes of *Prochlorothrix hollandica*. The gel was loaded with peak activity fractions from the sucrose density gradient centrifugation shown in Fig. 1. Gels were loaded with 20 μg (left hand) and 40 μg (right hand) of protein. (b) SDS-PAGE of dissociated purified *P. hollandica* RuBisCO. LSU, large subunit; SSU, small subunit.

Catalytic and regulatory properties

Maximum enzyme activity was achieved at pH 8.0 in HCO^3- and Mg^{2+}-supplemented Bicine buffer (Table I). Little or no activity was found above or below this value.

Divalent cations were a prerequisite for enzyme activity. Maximum activity was achieved in the presence of Mg^{2+} at 10 mM. Mg^{2+} could only partially be replaced by other divalent cations (Table II). Concentrations of these others at 10 mM by comparison with 5 mM had a marked inhibitory effect with activity being in the region of that obtained in the absence of divalent cations.

Sequence analysis

The sequence of 17 residues at the amino-terminus of the *P. hollandica* RuBisCO LSU was determined. The ninth amino acid was ambiguous but

Table I. Determination of pH optimum for the carboxylase activity of RuBisCO purified from *Prochlorothrix hollandica* carboxysomes.

pH	Specific activity[a]
7.5	0.0
7.7	0.0
7.9	0.05
8.0	0.56
8.1	0.15
8.3	0.05
8.5	0.03

[a] μmol CO_2-fixed $min^{-1} \cdot mg\ protein^{-1}$. Values are the means of duplicate determinations.

Table II. Effect of divalent cations on the carboxylase activity of RuBisCO purified from *Prochlorothrix hollandica* carboxysomes.

Assay conditions	Specific activity[a]	Relative activity [%]
10 mM Mg^{2+}	0.275	100.0
5 mM Mg^{2+}	0.163	59.3
Minus Mg^{2+}	0.007	2.8
Minus Mg^{2+}, plus 10 mM Mn^{2+}	0.009	3.3
Minus Mg^{2+}, plus 5 mM Mn^{2+}	0.066	24.0
Minus Mg^{2+}, plus 10 mM Co^{2+}	0.003	1.1
Minus Mg^{2+}, plus 5 mM Co^{2+}	0.071	26.0
Minus Mg^{2+}, plus 10 mM Ca^{2+}	0.003	1.1
Minus Mg^{2+}, plus 5 mM Ca^{2+}	0.079	29.0
Minus Mg^{2+}, plus 10 mM Cu^{2+}	0.007	2.8
Minus Mg^{2+}, plus 5 mM Cu^{2+}	0.08	30.0

[a] μmol CO_2-fixed $min^{-1} \cdot mg\ protein^{-1}$. Values are the means of triplicate determinations.

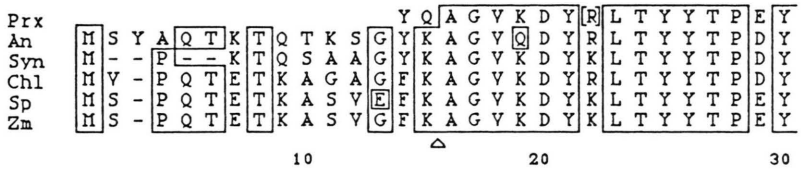

Fig. 3. Comparison of the N-terminal amino acid sequence of the LSU of *Prochlorothrix hollandica* RuBisCO (Prx), with sequences from the cyanobacteria *Anabaena* 7120 (An) and *Synechococcus* 6301 (Syn), and chloroplasts of *Chlamydomonas reinhardtii* (Chl), spinach (Sp) and maize (Zm). The single letter amino acid code is used; numbering the residues refers to the *Anabaena* sequence. *P. hollandica* Arg-22 (bracketed) is a tentative assignment. Positions at which four or more residues are identical are boxed. The cyanobacterial and chloroplast sequences are predicted primary translation products derived from gene sequences. The arrow indicates the putative proteolytic processing site. Sequences are taken from ref. [33–37].

arginine was the most likely of the possible residues. Comparison of the *P. hollandica* sequence with cyanobacterial and chloroplast sequences (Fig. 3) shows a high degree of conservation over the region sequenced, twelve amino acids being identical in all six species and two substitutions (at residues 22 and 29, numbering with respect to the *Anabaena* sequence) being conservative. The *Prochlorothrix* sequence shares identities with higher plant chloroplasts to the exclusion of cyanobacteria at some sites (Glu-29) but at others (Tyr-14) shows identity specifically with the cyanobacterial sequences.

Discussion

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from the prochlorophyte *Prochlorothrix hollandica* was purified from the carboxysomal cell fraction using vertical sedimentation into a reorientated sucrose gradient as the most effective method. Purification of the enzyme from the cytoplasmic pool was impaired by the persistent presence of contaminating proteins throughout the purification procedure. Similar problems have been encountered when purifying RuBisCO from photosynthetic bacteria [28, 29]. The latter groups resolved this problem by interrupting the vertical sedimentation gradient before the proteins had reached density equilibrium. Some pigment contaminants were separated from cytoplasmic *P. hollandica* RuBisCO using this approach, but this did not remove the major contaminating proteins when applied to the prochlorophyte enzyme. Other methods were tried such as gel filtration followed by FPLC, but enzyme preparations still contained undesirable yellow material. The problem was only resolved by separately purifying the soluble and particulate enzyme fractions, and assuming that the two enzyme forms are identical as in cyanobacteria [22, 23].

Particulate (carboxysomal) RuBisCO was purified to apparent homogeneity as determined by PAGE. The enzyme was composed of two types of subunits of MW 57 kDa and 13 kDa representing the large and small subunits of RuBisCO. These values are comparable to those estimated for the subunits of *Prochloron* of 57.5 kDa and 18.8 kDa respectively [17]. The MW of the native enzyme is within the range of those reported for similar 8L8S forms from higher plants and other groups of autotrophic organisms [15, 23].

The pure enzyme exhibited high specific activity (0.79 μmol CO_2-fixed $min^{-1} \cdot mg\ protein^{-1}$) but a yield of only 8% of the total cellular activity was obtained. However, on the basis of recovery from the original carboxysome-containing crude particulate fraction, a 20% yield was obtained. We have previously shown by immunoelectron microscopy and *in vitro* enzyme activity localization that RuBisCO is present both in the carboxysome and cytoplasmic fractions in *P. hollandica* and *Prochloron* using antiserum raised against the purified enzyme from a cyanobacterial source and that the prochlorophyte RuBisCO's are immunologically homologous with RuBisCO protein from cyanobacterial sources [18, 21].

The LSU sequence data are clearly too short to allow construction of a reliable phylogeny for the prochlorophyte. There is evidence that LSU processing occurs in chloroplasts [30], proteolytic cleavage between Lys-15 and Ala-16 (the site arrowed in Fig. 3) removing the first 14 residues to leave the mature protein with alanine at the N-terminus [31]. In *Prochlorothrix* the highly con-

served Lys–Ala pair of this putative processing site is replaced by the sequence Gln–Ala (a non-conservative substitution) and the mature protein is extended at the N-terminus by two residues, starting with tyrosine not alanine. The *Prochlorothrix* LSU may therefore be processed differently from the chloroplast protein.

RuBisCO from *P. hollandica* appears to be similar in structure to higher plant and cyanobacterial RuBisCOs. However, with respect to regulatory properties, the enzyme was not capable of using divalent cations other than Mg^{2+} for activation. It has been demonstrated in the photosynthetic bacterium *Rhodospirillum rubrum* that RuBisCO selectively catalyzes the oxygenase reaction when activated with Co^{2+} or Cu^{2+}, Mn^{2+} also had the same effect but to a lesser extent [27]. It is not known if this is the case in prochlorophytes but it could account for the low carboxylase activities obtained with the *P. hollandica* RuBisCO in the presence of these cations. This may reflect a more basic enzyme with respect to its regulatory properties. Further studies on the regulatory properties of prochlorophyte RuBisCO's and of the RuBisCO genes in these organisms may contribute to the understanding of the phylogeny of this interesting group, also now termed oxychlorobacteria [7], and of the molecular evolution of autotrophy.

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