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RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE FROM THE THERMOPHILIC, ACIDOPHILIC ALGA, *CYANIDIUM CALDARIUM* (GEITLER)

PURIFICATION, CHARACTERISATION AND THERMOSTABILITY OF THE ENZYME

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Summary

As an initial stage in the study of proteins from thermophilic algae, the enzyme ribulose 1,5-bisphosphate carboxylase 2-phospho-D-glycerate carboxylyase (dimerizing, EC 4.1.1.39) was purified 11-fold from the thermophilic alga *Cyanidium caldarium*, with a 24% recovery. This purified enzyme appeared homogeneous on polyacrylamide gels and could be dissociated into two subunit types of molecular weights 55 000 and 14 900. The optimal assay temperature was 42.5°C, whilst enzyme purified from *Chlorella* spp. showed maximum activity at 35°C. The thermostability of *Cyanidium* ribulose 1,5-bisphosphate carboxylase was considerably greater than that of the *Chlorella* enzyme, and the presence of Mg^{2+} and HCO_3^- further enhanced this heat stability. A break in the Arrhenius plot occured at 20°C for *Chlorella* ribulose 1,5-bisphosphate carboxylase and at 36°C for the enzyme from *Cyanidium*. It is suggested that the thermostability of *Cyanidium* ribulose 1,5-bisphosphate carboxylase is a result of an inherent stability of the enzyme molecule which permits efficient CO_2 fixation at high temperatures but results in low activity in the mesophilic temperature range.

Introduction

Cyanidium caldarium (Geitler) is a unicellular, eukaryotic alga occurring naturally in hot, acid springs at temperatures up to 57°C [1] and a pH of

between 1 and 2 [2]. Laboratory cultures of *Cyanidium* show optimal growth at 45—50°C [1,3] and optimal photosynthetic activity at 45—55°C [1,4—7].

It has been suggested by Singleton and Amelunxen [8] that the survival of thermophilic organisms is due primarily to an inherent stability of their macromolecules, in particular their cellular proteins. The enzyme ribulose 1,5-bisphosphate carboxylase (3-phospho-D-glycerate carboxy-lyase, EC 4.1.1.39) was selected as a suitable protein for an investigation of possible thermostability since this is the major CO_2-fixation enzyme in *Cyanidium* [9], and photosynthesis shows maximal activity at the high optimal growth temperature of the alga.

When examining the thermostability of the *Cyanidium* enzyme it was desirable to have available, for comparison, ribulose 1,5-bisphosphate carboxylase from a similar but typically mesophilic alga. The enzyme was purified from *Chlorella* for this purpose.

Materials and Methods

Materials

Cyanidium caldarium (Geitler) strain 1355/1 and *Chlorella* spp. strain 211/8P were obtained from the Culture Centre of Algae and Protozoa (Cambridge).

Ribulose 1,5-bisphosphoric acid (tetrasodium salt), bovine serum albumin and Tris (Trisma Base, reagent grade) were obtained from Sigma London Chemicals (Poole, Dorset), $NaH^{14}CO_3$ solution (specially purified for enzyme assays) was supplied by The Radiochemical Centre (Amersham, Bucks.) and DE-32 microgranular anion exchanger by Whatman (Maidstone, Kent). Acrylamide for electrophoresis was purchased from V.A. Howe (London), and standard proteins for calibration of acrylamide gels from Boehringer Corporation (London) (Lewes, Sussex). All other chemicals (Analar grade wherever possible) were obtained from British Drug Houses (Poole, Dorset).

Methods

Algal culture. Cyanidium culture medium contained 2.2 mM KH_2PO_4, 0.7 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 11.4 mM $(NH_4)_2SO_4$ and 0.04 mM $FeSO_4 \cdot 7H_2O$ (chelated with EDTA) in glass-distilled water. To each litre of culture medium was added 1 ml trace element solution (8 mM H_3BO_3, 2 mM $MnCl_2 \cdot 4H_2O$, 0.2 mM $ZnSO_4 \cdot 7H_2O$, 0.08 mM $CuSO_4 \cdot 5H_2O$, 0.04 mM $VOSO_4$, 0.03 mM $CoCl_2 \cdot 6H_2O$ and 0.007 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$). The medium was adjusted to pH 1.8 with H_2SO_4, and then autoclaved at 104 kPa.

Culture medium for *Chlorella* contained 5.6 mM KH_2PO_4, 1.4 mM K_2HPO_4, 1.6 mM $MgSO_4 \cdot 7H_2O$, 19.8 mM KNO_3 and 0.18 mM $FeSO_4 \cdot 7H_2O$ (chelated with EDTA) in glass-distilled water. To each litre of culture medium was added 1 ml trace element solution (46 mM H_3BO_3, 9 mM $MnSO_4 \cdot 4H_2O$, 0.8 mM $ZnSO_4 \cdot 7H_2O$, 0.6 mM $CuSO_4 \cdot 5H_2O$ and 0.02 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$). The medium was autoclaved at 104 kPa.

Culture bottles were inoculated with a suspension of algal cells and aerated with sterile, humidified air, enriched with 2% CO_2 for *Cyanidium* and 0.5% CO_2 for *Chlorella*. Addition agitational of the *Cyanidium* culture by a magnetic

stirrer was necessary to prevent the cells clumping and settling out. Illumination was provided.

Purification of ribulose 1,5-bisphosphate carboxylase from Cyanidium. *Cyanidium* cultures were harvested using an MSE Continuous Action Rotor at 2500 × *g* in an MSE 18 centrifuge. The resulting algal film was resuspended in the culture medium remaining in the rotor and the cells pelleted by centrifugation at 3000 × *g* for 10 min at 10° C. After washing with 0.05 M Tris-HCl (pH 8), the cells were resuspended in a small volume of the same buffer. The purification stages which follow were all carried out at 4° C unless otherwise stated. The algal cells were broken by passage through a French pressure cell and the resulting homogenate centrifuged at 100 000 × *g* for 1 h. The clear blue-green supernatant was used as a source of ribulose 1,5-bisphosphate carboxylase.

The precipitate forming at 40—50% saturation $(NH_4)_2SO_4$ was prepared and redissolved in 0.05 M Tris-HCl (pH 8). After dialysis overnight against the same buffer, the solution was loaded on to a column (20 × 3 cm) of DEAE cellulose (Whatman DE-32) previously equilibrated with 0.05 M Tris-HCl (pH 8). Proteins were eluted by a linear gradient of 0—0.3 M NaCl in the same buffer. The eluate was monitored for protein at 280 nm using an LKB Uvicord II.

DE-32 fractions containing ribulose 1,5-biphosphate carboxylase activity were pooled. The precipitate forming at 0—60% saturation $(NH_4)_2SO_4$ was prepared and dialysed against 0.05 M Tris-HCl (pH 8).

Purification of ribulose 1,5-bisphosphate carboxylase from Chlorella. The purification method reported above for *Cyanidium* was used exactly as described for the purification of ribulose 1,5-bisphosphate carboxylase from *Chlorella*.

Ribulose 1,5-bisphosphate carboxylase assay. The reaction mixture for the estimation of ribulose 1,5-bisphosphate carboxylase activity contained 125 μmol Tris-HCl (pH 8), 5 μmol $MgCl_2$, 15 μmol $NaH^{14}CO_3$ (1.5 μCi ^{14}C) and 0.4 mg enzyme protein in a total volume of 0.7 ml. After incubation at the assay temperature for 5 min, the reaction was initiated by the addition of 0.2 ml 2.5 mM ribulose 1,5-bisphosphoric acid. The reaction was allowed to proceed for 10 min at the selected temperature and then terminated by the addition of 0.2 ml 50% trichloroacetic acid. This acidification released any unfixed ^{14}C as $^{14}CO_2$. The reaction mixture was left overnight to allow complete release of unfixed ^{14}C. A reaction blank was prepared simultaneously where 0.2 ml ribulose 1,5-bisphosphoric acid was replaced by 0.2 ml 0.5 M Tris-HCl (pH 8). Duplicate 0.5 ml samples of the reaction mixtures were mixed with 2 ml scintillant [10]. Radioactivity was measured in a Nuclear Enterprises Model 8312 liquid scintillation counter at 90% efficiency.

Protein assay. Protein concentration was measured by the method of Lowry et al. [11] using bovine serum albumin as standard.

Electrophoresis. Purified preparations of ribulose 1,5-bisphosphate carboxylase from *Cyanidium* were examined for homogeneity by electrophoresis on 7.5% polyacrylamide gels using the method of Davis [12] but omitting the large pore spacer gel. Gels were stained for protein by immersion in 0.25% Coomassie Brilliant Blue in $CH_3COOH/CH_3OH/H_2O$ (1 : 5 : 5, v/v) for 1 h. Unbound stain was removed by batch destaining in regular changes of a solution containing $CH_3COOH/CH_3OH/H_2O$ (3 : 2 : 25, v/v). Dissociation of the

Cyanidium enzyme was achieved by incubation with 1% SDS and 1% 2-mercaptoethanol for 1 h at 70° C. The sub-units were separated by electrophoresis on 10% polyacrylamide gels using the system of Laemmli [13] at pH 8.3. Gels were stained with 0.25% Coomassie Brilliant Blue for 3 h and unbound stain removed as described above. Calibration gels containing 15—20 μg bovine serum albumin, catalase, aldolase, ovalbumin, chymotrypsinogen or cytochrome *c* were run simultaneously.

Results

Purification of ribulose 1,5-bisphosphate carboxylase from Cyanidium

An 11-fold purification of ribulose 1,5-bisphosphate carboxylase was achieved with a 24% recovery (Table I). Dialysis was found to be necessary following $(NH_4)_2SO_4$ fractionation, since NH_4^+ inhibited ribulose 1,5-bisphosphate carboxylase activity. It was also apparent that dialysis resulted in higher recovery of enzyme than desalting on a short Sephadex G-25 column. The elution profile from the DE-32 column showed an early peak of unbound protein, followed by a second peak of protein eluted by 0.1—0.12 M NaCl which was found to possess ribulose 1,5-bisphosphate carboxylase activity. Phycocyanin was eluted later by 0.17—0.19 M NaCl.

The purified enzyme appeared to be essentially homogeneous when examined by polyacrylamide gel electrophoresis (Fig. 1A). Dissociation of the native ribulose 1,5-bisphosphate carboxylase protein by SDS and mercaptoethanol, resulted in two sub-unit types of differing molecular weight (Fig. 1B). Comparison of the electrophoretic mobility of these two subunits with proteins of known molecular weight gave estimates of 55 000 and 14 900 for the molecular weights of the large and small sub-units respectively.

Ribulose 1,5-bisphosphate carboxylase from *Cyanidium* shows maximum activity at 42.5° C compared with an optimal assay temperature of 35° C for the enzyme purified from *Chlorella* (Fig. 2). The temperature profile for *Cyanidium* ribulose 1,5-bisphosphate carboxylase suggests a greater enzyme thermostability, though the enzyme from *Chlorella* is clearly more active at temperatures less than 35° C. Arrhenius plots of $\log_{10}k$ against reciprocal of absolute temperature for the two enzymes are shown in Fig. 3. Both enzymes

TABLE I

PURIFICATION OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE FROM *CYANIDIUM*

Step	Units of enzyme of activity (nmol CO_2 fixed · min^{-1})					
	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units · mg^{-1})	Purification (-fold)	Yield (%)
1. Crude Extract	30	1050	476	2.21	1.0	100
2. 40—50% $(NH_4)_2SO_4$	11	567	76.2	7.44	3.4	54
3. DE-32 Chromatography	29	251	13.6	18.5	8.4	24
4. 0—60% $(NH_4)_2SO_4$	5	248	10.4	23.8	10.8	24

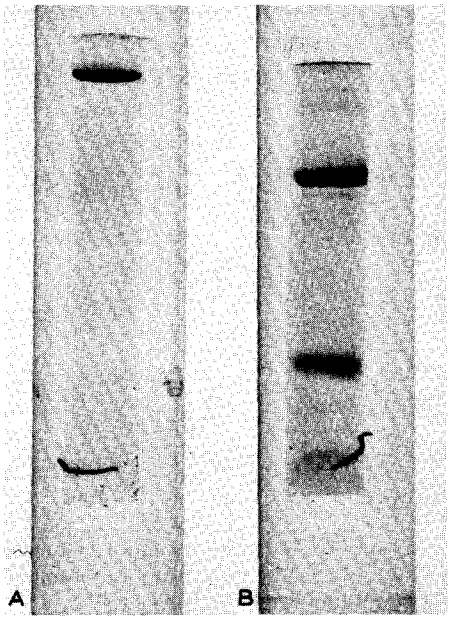


Fig. 1. Electrophoresis of *Cyanidium* ribulose 1,5-bisphosphate carboxylase on polyacrylamide gels. A. Electrophoresis of 20 μg native protein on 7.5% polyacrylamide. B. Electrophoresis of 25 μg enzyme protein on 10% polyacrylamide following dissociation of the enzyme by treatment with SDS and mercaptoethanol.

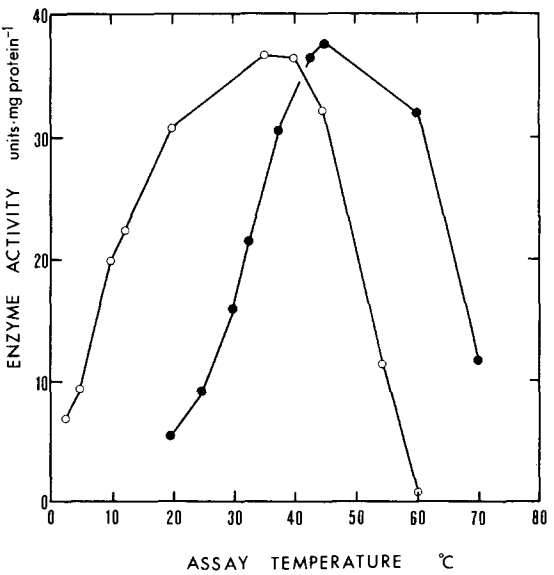


Fig. 2. Effect of temperature of assay on the activity of ribulose 1,5-bisphosphate carboxylase purified from either *Cyanidium* (●) or *Chlorella* (○). The enzyme was incubated for 5 min at the selected temperature, in a normal reaction mixture, before assay. Units of enzyme activity are nmol CO_2 fixed · min^{-1}.

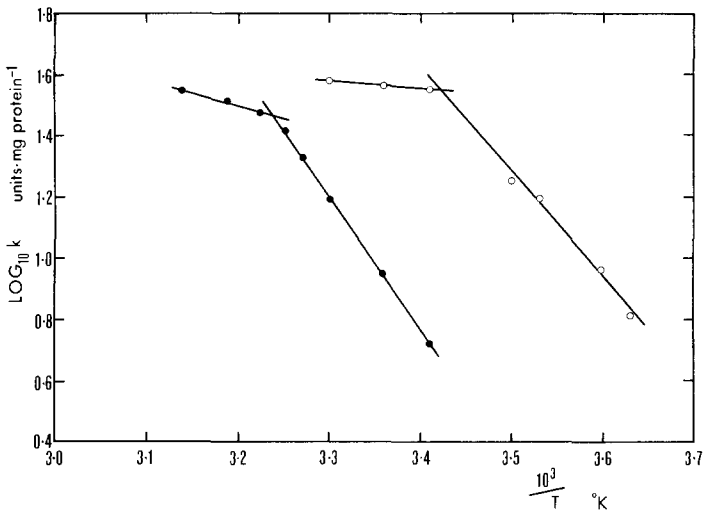


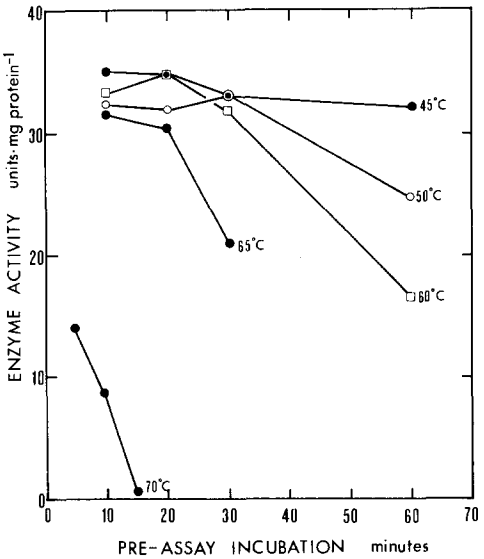
Fig. 3. Arrhenius plot of $\log_{10} k$ against reciprocal of absolute temperature (T) for ribulose 1,5-bisphosphate carboxylase purified from either *Cyanidium* (●) or *Chlorella* (○). Enzyme activity was measured using a pre-assay incubation period of 5 min. Units of enzyme activity are nmol CO_2 fixed · min^{-1}.

show a biphasic plot with a break occurring at 20° C for *Chlorella* ribulose 1,5-bisphosphate carboxylase and at 36° C for the *Cyanidium* enzyme. Activation energies, calculated from the slopes in Fig. 3, were 15 746 cal · mol^{-1} (2.5—20° C) and 1378 cal · mol^{-1} (20—30° C) for the *Chlorella* enzyme and 19 977 cal · mol^{-1} (20—36° C) and 3653 cal · mol^{-1} (36—45° C) for the enzyme from *Cyanidium*. The values for the *Chlorella* enzyme are both rather lower than the equivalent values for the *Cyanidium* ribulose 1,5-bisphosphate carboxylase. Both enzymes show an 80—90% reduction in activation energy at the break in the Arrhenius plot.

Thermostability of ribulose 1,5-bisphosphate carboxylase from Cyanidium and Chlorella

The thermostability of *Cyanidium* ribulose 1,5-bisphosphate carboxylase was examined by incubation of the enzyme, in a normal reaction mixture, over a range of temperatures for selected periods prior to initiation of the reaction with ribulose 1,5-bisphosphate. The results (Fig. 4) show little loss of activity after 1 h at 45° C. Increasing the temperature results in a progressive loss of activity, with time, but even after 1 h at 60° C, the enzyme still retains 51% of the activity shown at 45° C. Fig. 5 shows the results of similar experiments using ribulose 1,5-bisphosphate carboxylase purified from *Chlorella*. Although enzyme from this alga is also stable to 45° C treatment, it rapidly loses activity after 40 min at 50° C and is unable to tolerate temperatures in excess of 55° C.

The possibility of the presence of Mg^{2+} and HCO_3^- influencing the stability of ribulose 1,5-bisphosphate carboxylase was examined by repeating the above experiments but incubating the enzyme, prior to assay, in buffer alone. The results (Fig. 6) suggest that ribulose 1,5-bisphosphate carboxylase from *Cyanidium* is considerably less stable to heat in the absence of these two ions. Pre-



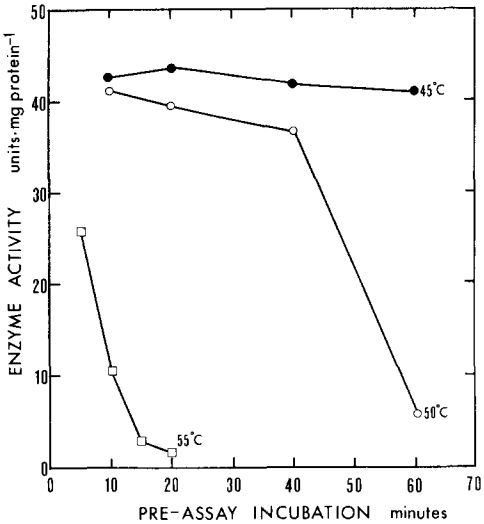


Fig. 4. Thermostability of ribulose 1,5-bisphosphate carboxylase, purified from *Cyanidium*, in the presence of Mg^{2+} and HCO_3^-. The enzyme was incubated, prior to assay, in normal reaction mixture for increasing periods of time at the temperatures described. At the end of each pre-assay incubation period, the remaining activity of the enzyme was estimated and expressed in units of nmol CO_2 fixed · min^{-1}.

Fig. 5. Thermostability of ribulose 1,5-bisphosphate carboxylase, purified from *Chlorella*, in the presence of Mg^{2+} and HCO_3^-. The enzyme was incubated, prior to assay, in a normal reaction mixture for increasing periods of time at the temperatures described. At the end of each pre-assay incubation period, the remaining activity of the enzyme was estimated and expressed in units of nmol CO_2 fixed · min^{-1}.

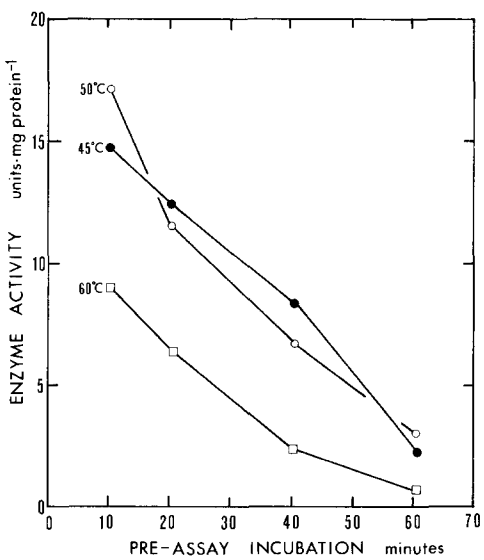


Fig. 6. Thermostability of ribulose 1,5-bisphosphate carboxylase purified from *Cyanidium*, in the absence of Mg^{2+} and HCO_3^-. The enzyme was incubated, prior to assay, in buffer alone for increasing periods of time at the temperatures described. At the end of each pre-assay incubation period, the necessary amounts of $MgCl_2$ and $NaH^{14}CO_3$ were added before initiating the reaction with ribulose 1,5-bisphosphoric acid. The enzyme activity remaining at the end of each pre-assay incubation period was estimated and expressed in units of nmol CO_2 fixed · min^{-1}.

TABLE II

THERMOSTABILITY OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE PURIFIED FROM EITHER *CHLORELLA* OR *CYANIDIUM*, IN THE PRESENCE AND ABSENCE OF Mg^{2+} AND HCO_3^-

The enzyme was incubated in the reaction mixture described below for 40 min at 50°C. The missing constituents were added just prior to initiating the reaction with ribulose 1,5-bisphosphate. Remaining enzyme activity was estimated and expressed either in units of nmol CO_2 fixed · min^{-1} · mg^{-1} protein or as a percentage of the activity in the total pre-assay incubation mixture.

Pre-assay incubation mixture *	Enzyme activity			
	Cyanidium		*Chlorella*	
	Units	%	Units	%
Mg^{2+} + HCO_3^-	31.1	100	5.7	100
Mg^{2+}	27.1	87	0.6	11
HCO_3^-	18.4	59	0.2	4
None	6.6	21	0.1	2

* Enzyme + buffer + additions.

assay incubation at 45, 50 or 60° C results in an almost linear decay in activity with time. The enzyme from *Chlorella* showed a similar decay in activity at 45° C but little stability to temperatures of 50° C or greater.

The effect of the presence of Mg^{2+} or HCO_3^- on the thermostability of ribulose 1,5-bisphosphate carboxylase from *Cyanidium* and *Chlorella* is shown in Table II. The protective ability of both these ions is evident and, of the two, Mg^{2+} is more effective at maintaining the stability of ribulose 1,5-bisphosphate carboxylase at high temperature. Although some protection against heat denaturation of the *Chlorella* enzyme is afforded by the presence of either Mg^{2+} or HCO_3^-, single ion enhancement of thermostability is much less effective with *Chlorella* ribulose 1,5-bisphosphate carboxylase than it is with the enzyme from *Cyanidium*.

Discussion

The enzyme ribulose 1,5-bisphosphate carboxylase has been purified and characterised from a number of prokaryotic and eukaryotic mesophilic algae. The molecular weight of the enzyme varies from 460 000 for *Anabaena cylindrica* [14] to 530 000 for *Chlorella fusca* [15]. On the basis of an 8 large plus 8 small sub-unit configuration for the native protein, a figure of 559 000 could be calculated for the molecular weight of ribulose 1,5-bisphosphate carboxylase from *Cyanidium*. In common with the enzyme from other sources it consists of two types of sub-units, the molecular weights of which fall within the range reported for other algae [15—18]. This close physical similarity between ribulose 1,5-bisphosphate carboxylase from the thermophilic alga *Cyanidium* and mesophilic algae, particularly *Euglena* [16] and *Chlamydomonas reinhardii* [17], would suggest that any structural modification of the enzyme for operation at elevated temperatures must be of a subtle nature.

The in vitro temperature optima for ribulose 1,5-bisphosphate carboxylase from *Cyanidium* and *Chlorella* are not markedly different and both enzymes show high stability to 45° C treatment in the presence of Mg^{2+} and HCO_3^-. How-

ever the enzyme from *Cyanidium* shows a much greater stability to temperatures of 50° C and higher. Nitrate reductase purified from *Cyanidium* was also found to be more stable to heat than the same enzyme from mesophilic algae [19]. Inherent heat stability of an enzyme molecule can only be demonstrated by subjecting the enzyme to high temperatures in the absence of co-factors or stabilising ions. Under such conditions, *Cyanidium* ribulose 1,5-bisphosphate carboxylase has been shown to possess a greater inherent thermostability than the enzyme from *Chlorella*. The presence of Mg^{2+} or HCO_3^- enhances the thermostability of enzyme from both sources, but to a much greater extent with *Cyanidium* ribulose 1,5-bisphosphate carboxylase, where Mg^{2+} is the more effective stabilising ion. The binding of Mg^{2+} to yeast transfer RNA specific for phenylalanine also resulted in increased thermostability of the molecule [20].

The superior thermostability of ribulose 1,5-bisphosphate carboxylase from *Cyanidium* appears to be correlated with a higher temperature requirement for CO_2 fixation. Comparison of Arrhenius plots shows a break at 20° C for the *Chlorella* enzyme and 36° C for the enzyme from *Cyanidium*. A similar situation has been reported for 6-phosphogluconate dehydrogenase from *Penecillium notatum* (mesophile) and *P. duponti* (thermophile) [21], and ribose-5-phosphate isomerase from mesophilic and thermophilic bacteria [22]. It has been suggested that such a break in an Arrhenius plot denotes a conformational change in the enzyme molecule. For ribulose 1,5-bisphosphate carboxylase this conformational change markedly reduces the activation energy required for the reaction.

The inherent thermostability of *Cyanidium* ribulose 1,5-bisphosphate carboxylase contrasts with that of a trehalase isolated from the thermophilic fungus *Humicola lanuginosa* [23]. In this organism the ability of trehalase to retain activity at high temperature was ascribed to the presence of proteinaceous stabilising factors in the cell. Ribulose 1,5-bisphosphate carboxylase from *Cyanidium* would also appear to differ from soluble proteins (extracted in two bulk fractions) from this alga which did not show superior heat stability to soluble proteins extracted by the same method from four mesophilic algae [24]. Also cytochrome *c*-553 purified from *Cyanidium*, whilst showing high thermostability, may not differ in this respect from the same protein from mesophilic algae [25].

It would appear that *Cyanidium* possesses a ribulose 1,5-bisphosphate carboxylase suitable for efficient photosynthesis at the high temperatures it encounters in nature, whereas the *Chlorella* enzyme has become adapted to operate with greater efficiency in the mesophilic temperature range and has thereby lost its stability to high temperature.

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