Purification, Characterization and Thermostability of Ribulose 1,5-Bisphosphate Carboxylase-Oxygenase from Barley Leaves

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Z. Naturforsch. **55c,** 611–619 (2000); received September 21, 1999/February 9, 2000

Rubisco, Ribulose-1,5-bisphosphate Carboxylase-Oxygenase, N-Terminal Sequence, Thermostability

The enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (rubisco) and its functional subunits from barley (*Hordeum vulgare* L.) leaves were purified to homogeneity by activity-directed sequencial steps of chromatography. Based on the molecular mass estimation by SDS-PAGE, the large subunit (LS) had an apparent molecular weight of *ca.* 55 kDa, whereas the small subunit (SS) was *ca.* a 14 kDa polypeptide chain. The N-terminal sequences, established by automated Edman degradation analysis of the purified subunits, showed very close sequence homologies (52–92%) with the subunits of other rubisco enzymes reported from several photosynthetic species. In order to establish the chemical heterogeneity in the rubisco from barley, the amino acid composition of purified native enzyme was analyzed and the results systematically compared with other known type-I rubisco enzymes from spinach, maize, tobacco and pea. Major differences have been observed in the amino acid composition of barley rubisco, the concentration of cysteine, serine, threonine, isoleucine, leucine, arginine and tryptophan residues were found quite variable as compared to other higher plants. The thermostability of the native rubisco was also investigated using circular dichroism and fluorescence spectroscopy. The critical (T_c) and melting (T_m) temperatures were determined to be 60 °C and 57 °C, respectively, and at this temperature the enzyme not only retains its structural integrity but also its enzymatic activity. Results of these studies were discussed in the light of structural and functional adaptation of this bifunctional enzyme in C_3 and C_4 plants to their environments.

Introduction

Ribulose-1,5-bisphosphate carboxylase-oxygenase (rubisco, EC 4.1.1.39) is the most abundant class of soluble proteins in the leaves of plants. The importance of rubisco in photosynthesis and photorespiration makes it a major object for many structural and functional studies (for review see, Schneider *et al.*, 1992; Hartman and Harpel, 1994; Wildner *et al.*, 1996). Due to the complex architecture and very high molecular mass of native rubisco, most of the structural details available are based on electron microscopy, X-ray crystallography and gene sequence analysis. On the basis of structural considerations rubisco enzymes are divided into two major groups. The enzyme from the nonsulfur purple bacterium *Rhodospirillum rubrum* (Andersson *et al.*, 1989) consists of only two large subunits and is termed as type II rubisco. In contrast, the type I rubisco belongs to cyanobacteria, green algae and higher plants, and is a complex of eight large subunits and eight small subunits (Hartman and Harpel, 1994; Taylor and Andersson, 1997).

Abbreviations: CD, circular dichroism; FPLC, fast protein liquid chromatography; NAD/NADH nicotinamide adenine dinucleotide (oxi/red); RP HPLC, reverse phase high performance liquid chromatography; Rubisco, ribulose-1,5-bisphosphate carboxylase-oxygenase (EC 4.1.1.39); SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Tris, tris (hydroxymethyl) aminomethane.

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The crystal and the gene structures of many higher plant rubisco enzymes and its subunits have been well established with spinach (*Spinacia oleracea* L., Taylor and Andersson, 1997; Zurawski *et al.*, 1981; Knight *et al.*, 1989), tobacco (*Nicotiana tabacum* L., Shinozaki and Sugiura, 1982; Mazur and Chui, 1985; Chapman *et al.*, 1987; Chapman *et al.*, 1988), maize (*Zea mays* L., McIntosh *et al.*, 1980; Zurawski *et al.*, 1984), pea (*Pisum sativum* L., Zurawski *et al.*, 1986) and the partial gene sequence of the large subunit from barley (*Hordeum vulgare* L., Zurawski *et al.*, 1984). Results of these studies revealed that in most of the photosynthetic organisms, including higher plants, rubisco is a hexadecamer (*Mr.* 560 kDa) consisting of eight large (LS) and eight small (SS) subunits in an L_8S_8 arrangement. All investigated plants have an L_8S_8 rubisco structure with high sequence homology *ca.* 80% among the large catalytic subunit and *ca.* 70% in the small subunit with some still unknown function (Knight *et al.*, 1989). The complete amino acid sequence of rubisco large subunit comprises of 475 amino acid residues (50–55 kDa) and about 123 amino acid residues (12–14 kDa) in the small subunit. Furthermore, sequence comparison between the two types of rubisco enzymes, namely higher plants type I and the purple bacteria type II rubisco, revealed only *ca.* 28% sequence homologies mainly around the two highly conserved sites suggesting an independent evolution of these two enzyme groups (for review see Miziorko, 1983).

In our previous studies we have demonstrated that despite the high degree of sequence homology (*ca.* 70–90%) among the subunits within type I (barley, spinach, wheat, maize, pea and tobacco) rubisco, immunological reactivity of monoclonal antibodies against different plant species are different (Simova *et al.*, 1998). That is why the study of different biological properties of rubisco enzymes is of prime interest to establish the protein structure-function relationship. In the present study we have performed isolation, purification and characterization of rubisco, and its two purified subunits from barley (*Hordeum vulgare* L.) leaves. The N-terminal sequences of the purified subunits, chemical composition of the native enzyme, and its stability towards temperature denaturation have been established.

Materials and Methods

Plant material

Green leaves from barley (*Hordeum vulgare* L.) plants variety "Hemus" were used. Pre-soaked barley seeds were germinated and the plants grown under photothermostatic conditions, 12 h photoperiod (180 $E.m^{-2}.s^{-1}$) at 27 °C during the day and 20–25 °C at night. After 10 days, the primary fully expanded leaves were harvested, and rapidly frozen in liquid nitrogen.

Isolation and purification of rubisco

Leaves (25 g), frozen in liquid nitrogen were ground to a fine powder and homogenised with 100 mM Tris[tris(hydroxymethyl)aminomethane]-HCl buffer, pH 8.0, containing 20 mM $MgCl_2.6H_2O$, 20 mM $NaHCO_3$, 1 mM ethylenediaminetetraacetic acid, 20 mM β-mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride, 3% w/v Polyclar AT, 1% w/v ovalbumin (before extraction) and 12.5% v/v glycerol. The homogenate was filtered through eight layers of cheese cloth containing a layer of cotton wool and then centrifuged for 30 min at 12000×*g*. The extract was precipitated with 35–55% $(NH_4)_2SO_4$ and centrifuged for 30 min at 16000×*g*. The pellet was redissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 20 mM $MgCl_2.6H_2O$, 1 mM EDTA, 20 mM β-mercaptoethanol and 12.5% v/v glycerol. The precipitate was preliminary fractionated on a column of Sephacryl S-200 (50 × 1,60 cm; flow rate of 15 ml/h) under the same buffering conditions. The eluted fractions enriched with enzymatically active rubisco (termed fraction-I) were purified on a DEAE-Sephacel anion-exchange column (6.5 × 3.4 cm; flow rate 45 ml/h) equilibrated under the same buffering conditions as described above. Anion-exchange chromatography with a stepwise gradient of KCl, 100 to 600 mM was employed. Rubisco was eluted with 300 mM KCl. The fractions were analyzed for protein at 585 nm according to Read and Northcote (1981). The enzymatically active fractions were pooled and dialysed gradually (10 times) against 5 mM Tris-HCl buffer, pH 8.0, lyophylized immediately and stored at −70 °C until further use.

The last purification step of rubisco was achieved by anion-exchange FPLC column

(Mono-Q 10/10) equilibrated with 50 mM Tris-HCl buffer, pH 8.0, and eluted with a linear gradient of 0 to 400 mM KCl in the same buffer. The eluate was monitored at 280 nm with a flow rate of 60 ml/h. All steps of isolation and purification of rubisco were performed in a cold room at 4 °C. The each step of purification was followed by total protein estimation (Bradford, 1976) and the enzymatic activity (Lan and Mott, 1991).

Rubisco subunit dissociation was made according to Gooding *et al.* (1973) with slight modifications. The enzyme protein (ca. 20 mg) was treated with p-chloromercuribenzoate at pH 9 for 30 min in the presence of 6 M guanidine hydrochloride and 0.18 M sodium sulfite, and separated into LS and SS on a Sephadex G-100 column (45 x 2.5 cm; flow rate 20 ml/h) containing 0.1 M sarcosine and 7 M urea. The protein fractions were pooled and the mercury groups were removed by incubation with 0.5 M β-mercaptoethanol and 2.7 mM ethylenediaminetetraacetic acid for 2 h at room temperature and dialysed against 10 mM Tris-HCl buffer, pH 8.0.

The salt-free pure subunits preparations for the N-terminal sequence and the native enzyme for amino acid composition analysis were achieved by HPLC using Nucleosil $7C_{18}$ reverse phase column (250 × 10 mm, Macherey-Nagel, Düren, Germany). The following conditions for separation was used: eluent A, 1% trifluoroacetic acid in water (v/v); eluent B, 80% acetonitrile in H_2O and 0.58% TFA (v/v/v); gradient program, 0–60% B in 60 min; flow rate, 2 ml/min. The eluate was monitored at 280 nm and lyophylized.

Assay of enzyme activity

Rubisco carboxylase activity was determined as described by Lan and Mott, (1991) using spectrophotometric methods including a cascade enzyme system with oxidised nicotinamide adenine dinucleotide (NAD), as the final product measuring NADH concentration at 340 nm and calculated on a stoichiometry of 2:1 between NADH oxidation and ribulose-1,5-bisphosphate carboxylation. Before assay the enzyme was activated by incubation for 10 min at 25 °C in 200 mM Tris-HCl buffer, pH 8.0, containing 40 mM $MgCl_2$, 0.4 mM EDTA, 2 mM dithiothreitol, and 20 mM $NaHCO_3$ in 1:1 ratio (v/v) of enzyme to the activation buffer. Enzyme activity was measured on Shimadzu spectrophotometer.

SDS polyacrylamide gel electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). The protein samples (*ca.* 50 μg), determined according to Bradford (1976) were loaded on 15% gel and stained with 0.2% Coomassie brilliant blue R-250 stain in 25% methanol, 10% acetic acid and water (w/v/v/v).

Amino acid sequence analysis

Automated N-terminal Edman degradation analysis was performed using an Applied Biosystems pulsed liquid sequencer model 473A (Weiterstadt, Germany) with on-line analysis of the phenylthiohydantoin derivatives. Approximately 100–200 pmol of purified samples were applied on the cartridge filter, previously treated with polybrene. The repetitive yield during sequencing of these subunits was 93–95%.

Amino acid analyses

Amino acid composition of the purifed native rubisco was performed after acid hydrolysis in 5.7 N HCl in evacuated sealed tubes for 24, 48 and 72 h at 110 °C. The hydrolysates were analysed with a Biotronik model LC 3000 automatic amino acid analyser (Germany). Tryptophan was determined in the presence of 6% thioglycolic acid. Oxidation of the protein with performic acid (1 vol. of 30% (w/w) H_2O_2 to 9 vol. of 88% (w/w) formic acid), converting cysteine and cystine into cysteic acid, was used for quantitative determination of these residues. The amounts of valine, isoleucine and leucine were calculated from 72-h samples.

Thermostability of rubisco

Circular dichroism measurements were performed by a dichrograph (Jasco J-720), equipped with a personal computer IBM PC-AT, PS/2, multiscan monitor CMS-3436 and a Hewlett-Packard colour graphics plotter model HP 7475A. A software DOS version was used for calculation of the CD data. Protein solution (*ca.* 0.1 mg/ml) in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$ was placed in sample holder thermostati-

cally controlled using a NESLAB thermostat model RTE-110 and connected with a digital programming controller. The samples were kept for 10 min at the desired temperature to ensure the attainment of thermal equilibrium which was confirmed by the constancy of the ellipticity. Each spectrum, used for further calculation, represents an average of two or three separate scans. The far-UV CD spectra were recorded between 200 and 260 nm.

The temperature dependence of the rubisco enzyme was also determined by fluorescence spectroscopy in 50 mM Tris-HCl buffer, pH 8.0, contaning 10 mM $CaCl_2$ using the spectrofluorimeter model LS5 (Perkin-Elmer) equipped with a thermostatically controlled assembly and a data station model 3600. The optical absorbance of the solutions was less than 0.05 at the excitation wavelength at 295 nm to avoid inner filter effects. The samples were kept for 10 min at the desired temperature prior to the measurements, to ensure thermal equilibration. The results were analyzed according to the Arrhenius equation (Kirby and Steiner, 1970),

$$\ln (Q\text{-}1\text{-}1) = -E_a/RT + \ln k$$

where, Q is the emission quantum yield, E_a (kJ.mol^{-1}) is the activation energy of the radiationless deactivation of the singlet excited state, R and k are constants.

Results and Discussion

The enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (rubisco) and its subunits have been purified to homogeneity from the barley (*Hordeum vulgare* L.) leaves by the activity – directed purification steps of $(NH_4)_2SO_4$ precipitaion, Sephacryl S-200, DEAE-Sephacel anion-exchange and Mono-Q fast protein liquid chromatography. Ammonium sulfate precipitation of the barley leaves extract resulted in about 87% recovery of the total rubisco enzymatic activity. Figure 1 depicts the purification profile of the native rubisco enzyme by DEAE-Sephacel anion-exchange chromatography. Full purification of the native rubisco was achieved by anion-exchange Mono-Q fast protein liquid chromatography (Fig. 2). Approximately 19 mg (total yield of 4.2%) of pure rubisco was obtained from 450 mg of total barley extract with specific rubisco carboxylase activity of 440 nmol. min^{-1}. mg^{-1}. Electrophoretic analysis of the obtained fractions suggest that the native rubisco consists of large (LS) and small subunits (SS) with approximate molecular masses of 55 and 14 kDa, respectively (Fig. 3). Further homogeneity and the N-terminal sequence analysis of the obtained subunits were

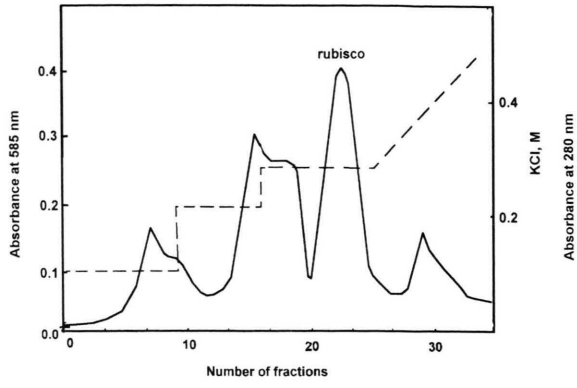

Fig. 1. Purification profile of ribulose 1,5-bisphosphate carboxylase-oxygenase from barley (*H. vulgare* L.) leaves on a DEAE-Sephacel anion-exchange column (6.5 × 3.4 cm; flow rate 45 ml/h), equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 20 mM $MgCl_2 \cdot 6H_2O$, 1 mM EDTA, 20 mM β-mercaptoethanol and 12.5% v/v glycerol, and eluted with a stepwise gradient of KCl (100 to 600 mM). The fractions were analyzed for protein at 585 nm.

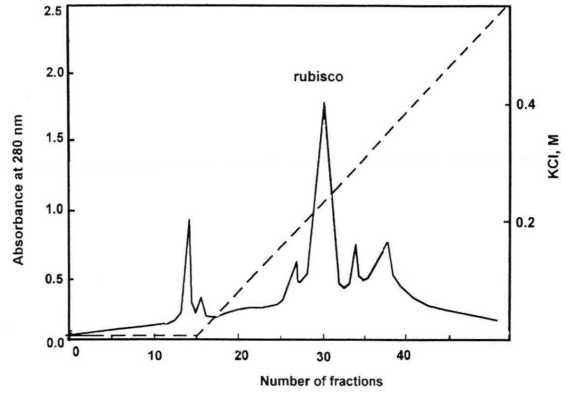

Fig. 2. Fast protein liquid chromatographic of ribulose 1,5-bisphosphate carboxylase-oxygenase from barley (*H. vulgare* L.) leaves. The anion-exchange column Mono-Q type (10/10) was equilibrated in 50 mM Tris-HCl buffer, pH 8.0, and eluted with a linear gradient of KCl in the same buffer (0–400 mM). The eluate was monitored at 280 nm with a flow rate of 60 ml/h.

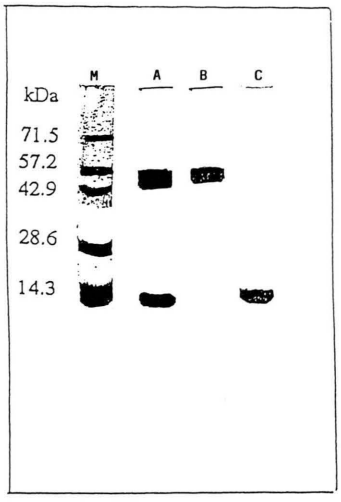

Fig. 3. SDS-PAGE analysis of ribulose 1,5-bisphosphate carboxylase-oxygenase and its purified subunits from barley (*H. vulgare* L.) leaves on 15% polyacrylamide gel. Tracks are loaded with the following samples: lane M, low molecular weight markers; lane A, rubisco; lane B, LS-large subunits and lane C, SS-small subunits.

established by automatic N-terminal Edman degradation analysis (Table I).

Comparison of the N-terminal sequences of the two purified large and small subunits (LS/SS) of rubisco reveals different percentage of sequence homologies. Major variation has been observed in the N-terminal sequence of barley rubisco small subunit, where the sequence analogy is restricted to only 44–60% in comparison to all other type-I rubisco enzymes (Table I). On the other hand, high degree of structural identities (84–92%) have been observed in the large subunits of all type-I (barley, spinach, wheat, maize, pea and tobacco) rubisco enzymes. At least three highly conserved structural motifs (i.e. Met^1 – Ala^9, $Ala1^5$ – Lys^{18} and Lys^{21} – Tyr^{25}) can be identified in the N-terminal sequences of all rubisco large subunits. Many point mutational studies on the native rubisco by constructing the chimeric genes suggest that the N-terminus of large subunit is very important in the catalytic activity of the enzyme, either directly in the substrate binding or in maintaining the integrity of the active site (Kettleborough *et al.*, 1987; 1991). Limited proteolysis studies on many type-I rubisco enzymes revealed the differential loss of carboxylase activities (e.g. 80% in wheat and spinach but not in barley) which correlated with the loss of a small variable peptide "Ala^9 to Q^{14}" between the two highly conserved motifs (Table I), while the small subunit was unaffected by this treatment (Gutteridge *et al.*, 1986). Deviation in the primary structure of barley rubisco in this protease susceptible peptide "Ala^9 to Q^{14}" where a Lys^{14} has been replaced with Gln^{14} confirmed the involvement of this N-terminal peptide in catalysis.

In order to establish the chemical heterogeneity in rubisco enzyme, the amino acid composition of the purified native enzyme was determined after acid hydrolysis. On the basis of available crystallographic (Andersson *et al.*, 1989; Knight *at al.*, 1989; 1990; Taylor and Andersson, 1997) and gene structures (McIntosh *et al.*, 1980; Zurawski *et al.*, 1981; Shinozaki and Sugiura, 1982; Zurawski *et al.*, 1986) which revealed the 123 residues for the small subunit and 475 amino acid residues for the large subunits we have calculated the amino acid compositions of other native rubisco enzymes and systematically compared them with barley rubisco (Table II). Major differences have been observed in the amino acid composition of barley rubisco, where the concentration of Cys, Ser, Thr, Ileu, Leu, Arg and Trp residues are quite variable as compared to the other higher plants. While the remaining amino acid residues in barley rubisco are in close proximity to those calculated for spinach, maize, tobacco and pea i.e. Gly, Ala, Met, Lys, Val, Asp, Asn, Glu, Gln, Pro, His, Phe, and Tyr.

The conformational stability toward thermal denaturation of the native rubisco enzyme was studied using CD spectroscopy. The far-UV CD spectrum of the native rubisco enzyme reflects the backbone conformation of the protein molecule. It reveals two major negative bands at 208 and 222 nm, characteristic for the α-helical structure. Around 216 nm, the β-sheet structure also contribute to the ellipticity of the α-helix bands. The CD spectrum, obtained for our enzyme, is very close to the other rubisco enzymes and the mean residue ellipticity at 208 and 222 nm was found to be 5400 and 4800 $deg.cm^2.dmol^{-1}$, respectively. The melting temperature of "T_m 57 °C" was calculated for the native enzyme (Fig. 4) from the variable temperature CD spectra.

The thermostability of the native rubisco enzyme was also complemented by fluorescence spectroscopy. The fluorescence spectra of the na-

Table I. Comparison of the N-terminal sequences of ribulose 1,5-bisphosphate carboxylase-oxygenase subunits from barley (*H. vulgare*) leaves. Rubisco large and small subunits sequences have been obtained from *S. oleracea* (Zurawski *et al.*, 1981; Knight *et al.*, 1990), *Z. mays* (Zurawski *et al.*, 1984; McIntosh *et al.*, 1980) *P. sativum* (Zurawski *et al.*, 1986; Fluhr *et al.*, 1986), *N. tabacum* (Shinozaki and Sugiura, 1982; Mazur and Chui, 1985) *T. aestivum* (Gutteridge *et al.*, 1986), *H. vulgare* (from this study). * The partial gene structure of the large subunit of barley rubisco was first described by Zurawski *et al.*, (1984).

A) Large subunits (LS)

	5								10								15								20								25
*H. vulgare**	M	S	P	Q	T	E	T	K	A	G	V	G	F	Q	A	G	V	K	D	Y	K	L	T	Y	Y
S. oleracea	M	S	P	Q	T	E	T	K	A	S	V	E	F	K	A	G	V	K	D	T	K	L	T	Y	Y
P. sativum	M	S	P	Q	T	E	T	K	A	S	V	G	F	K	A	G	V	K	D	Y	K	L	T	Y	Y
N. tabacum	M	S	P	Q	T	E	T	K	A	S	V	G	F	K	A	G	V	K	E	T	K	L	T	Y	Y
T. aestivum	M	S	P	Q	T	E	T	K	A	G	V	G	F	K	A	G	V	K	D	Y	K	-	T	Y	Y
Zea mays	M	S	P	Q	T	E	T	K	A	S	V	G	F	K	A	G	V	K	D	Y	K	L	T	Y	Y

B) Small subunits (SS)

	5								10								15								20								25
*H. vulgare**	M	Q	V	W	D	P	I	G	K	K	F	K	D	T	K	S	-	L	P	L	L	T	A	S	N
S. oleracea	M	Q	V	W	P	P	L	G	L	K	K	F	E	T	L	S	Y	L	P	P	L	T	T	E	Q
P. sativum	M	Q	V	W	P	P	I	G	K	K	K	F	E	T	L	S	Y	L	P	P	L	T	R	D	Q
N. tabacum	M	Q	V	W	P	P	I	N	K	K	K	Y	E	T	L	S	Y	L	P	D	L	S	Q	E	Q
T. aestivum	M	Q	V	W	P	I	E	G	I	K	K	F	E	T	L	S	Y	L	P	P	L	S	T	E	A
Zea mays	M	Q	V	W	P	A	Y	G	N	K	K	F	E	T	L	S	Y	L	P	P	L	S	T	D	D

tive rubisco enzyme shows an emission maximum at 340 nm which is typical for most globular proteins, and used for the characterization of the tryptophan emission. The standard N-Ac-Trp-NH_2 with a λ_{max} 350 nm was used which is typical for exposed tryptophan residues. Our results suggest that the tryptophan residues in the native molecule are equally distributed and some of the tryptophan residues are exposed while others are buried in the hydrophobic environment of the protein molecule. These results are in good agreement with the published three-dimensional structure of spinach rubisco (Knight *et al.*, 1990). The thermal denaturation of rubisco, i.e. the unfolding process

Table II. Amino acid composition of native ribulose 1,5-bisphosphate carboxylase-oxygenase enzyme from barley (*H. vulgare* L.) leaves, compared with the amino acid composition of other rubisco enzymes from higher plants. * Values are calculated from the available crystal (Andersson *et al.*, 1989; Knight *at al.*, 1989; 1990; Taylor and Andersson, 1997) and the gene structures (McIntosh *et al.*, 1980; Zurawski *et al.*, 1981; Shinozaki and Sugiura, 1982; Zurawski *et al.*, 1986). AAs = amino acids.

AAs	Barley	Spinach*	Maize*	Tobacco*	Pea*
Gly	60	53	54	48	50
Ala	47	46	43	46	41
Cys	8	12	15	11	11
Ser	42	18	24	20	17
Met	11	12	13	11	12
Lys	28	32	32	29	27
Val	42	46	37	42	36
Thr	26	36	31	33	32
Ile	33	26	27	26	23
Leu	39	47	44	51	49
Asp	31	32	33	32	32
Asn	20	19	16	17	14
Glu	37	40	31	40	38
Gln	17	16	17	18	17
Arg	14	30	30	28	31
Pro	27	30	28	29	28
His	15	16	14	13	15
Phe	25	23	24	17	20
Tyr	27	28	23	27	23
Trp	8	12	12	13	13
Total	557	562	548	551	529

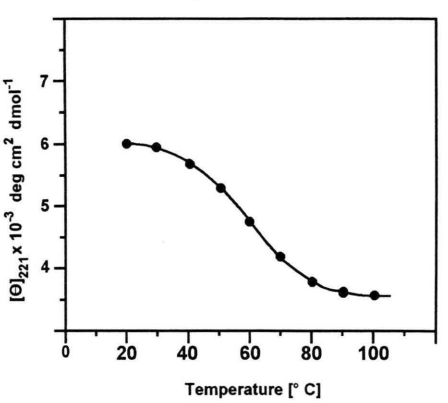

Fig. 4. Temperature dependence of the ellipticity [Θ] at 221 nm of ribulose 1,5-bisphosphate carboxylase-oxygenase from barley (*H. vulgare* L.) leaves. Measured by means of circular dichroism in 50 mM Tris/HCl buffer, pH 8.0, containing 10 mM $CaCl_2$. See text, Materials and Methods for details.

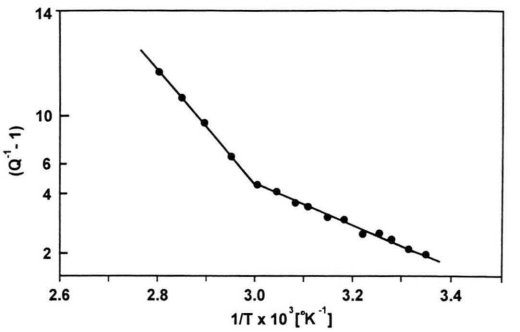

Fig. 5. Thermal dependence of the tryptophan fluorescence of ribulose 1,5-bisphosphate carboxylase-oxygenase from barley (*H. vulgare* L.) leaves. Measured by means of fluorometric determination of enzyme in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$. The fluorescence quantum yield was determined using N-Ac-Trp NH_2 as a standard. See Materials and Methods for details.

taking place after passing the critical temperature (T_c), was irreversible, and for this reason equilibrium thermodynamic parameters were not determined. We used the T_c-value, the critical temperature for deviation of the Arrhenius plot (ln (Q^{-1}-1) vs $1/T$) from the linearity, to characterize the thermostability of rubisco. The deviation indicates that at the temperature above T_c the protein undergoes denaturation. A typical Arrhenius plot for the native rubisco is shown in Figure 5. The activation energy for the thermal deactivation of the excited protein fluorophores, Ea, is also calculated for the native enzyme which was found to be 17 kJ.mol^{-1} . Results of these studies show that the rubisco enzyme is conformationally stable at temperature up to 60 °C.

The critical (T_c) and the melting (T_m) temperatures determined by the two methods to be 60 °C and 57 °C, respectively, and at the temperature above these values rubisco not only lose its structural integrity but also the enzymatic activity. Thermostability of the barley rubisco bifunctional enzyme (i.e. photosynthetic and photorespiratory functions) can also be well correlated with the functional studies reported for the same class of enzyme (Chollet and Anderson, 1976; Monson *et al.*, 1982; Ghosh *et al.*, 1989). Studies on C_4 and C_3 plants (such as; sorghum/corn, and peanut/soybean, respectively) shows that at 40–45 °C the native rubisco enzymatic activities and concentration level of rubisco subunits were significantly higher.

While at 50 °C the enzymatic activities were dropped 54% to 84% and the level of rubisco subunits either remained relatively stable (large subunits) or increased (small subunits) in plants native to hot climates (sorghum and peanut). Furthermore, in the plants native to temperate climates (corn and soybean) levels of the proteins either fell sharply (corn) or showed strong evidence of incomplete processing (soybean) and/or aggregation (Chollet and Anderson, 1976; Ghosh *et al.*, 1989). Rubisco from barley (*Hordeum vulgare* L.) variety "Hemus" can be classified as heat-stable, since full enzymatic activity and structural features were retained after treatment at 57–60 °C.

Acknowledgements

We express our gratitude to the Deutsche Forschungsgemeinschaft (DFG-Bonn, Germany) for granting a scholarship to Dr. Pavlina Dolashka-Angelova and Deutscher Akademischer Austauschdienst (DAAD, Bonn, Germany) for a scholarship (41-HP-cr-gb) to Dr. S. Abid Ali.

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