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Markedly low requirement of added CO_2 for photosynthesis by mesophyll protoplasts of pea (*Pisum sativum*): possible roles of photorespiratory CO_2 and carbonic anhydrase

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Mesophyll protoplasts of pea required only 74.1 μM CO_2 for maximal photosynthesis, unlike chloroplasts, which required up to 588 μM CO_2. Such a markedly low requirement for CO_2 could be because of an internal carbon source and/or a CO_2 concentrating mechanism in mesophyll protoplasts. Ethoxyzolamide (EZA), an inhibitor of internal carbonic anhydrase (CA) suppressed photosynthesis by mesophyll protoplasts at low CO_2 (7.41 μM) but had no significant effect at high CO_2 (741 μM). However, acetazolamide, another inhibitor of CA, did not exert as much dramatic effect as EZA. Three photorespiratory inhibitors, aminoacetonitrile or glycine hydroxamate (GHA) or aminooxyacetate inhibited markedly photosynthesis at low CO_2 but not at high CO_2. Inhibitors of glycolysis or tricarboxylic acid cycle (NaF, sodium malonate) or phosphoenolpyruvate carboxylase (3,3-dichloro-2-dihydroxy phosphinoyl-methyl-2-propenoate) had no significant effect on photosynthesis. The CO_2 requirement of protoplast photosynthesis and the sensitivity of photosynthesis to EZA were much higher at low oxygen (65 nmol ml^{-1}) than that at normal oxygen (212 nmol ml^{-1}). In contrast, the inhibitory effect of photorespiratory inhibitors on protoplast photosynthesis was similar in both normal and low oxygen medium. The marked elevation of glycine/serine ratio at low O_2 or in presence of GHA confirmed the suppression of photorespiratory decarboxylation by GHA. While demonstrating interesting difference between the response of protoplasts and chloroplasts to CO_2, we suggest that photorespiration could be a significant source of CO_2 for photosynthesis in mesophyll protoplasts at limiting CO_2 and at atmospheric levels of oxygen. Obviously, carbonic anhydrase is essential to concentrate or retain CO_2 in mesophyll cells.

Abbreviations – AAN, aminoacetonitrile; AOA, aminooxyacetate; ATP, adenosine triphosphate; AZA, acetazolamide; CA, carbonic anhydrase; CCM, carbon concentrating mechanism; DCDP, 3,3-dichloro-2-dihydroxy phosphinoyl-methyl-2-propenoate; EZA, ethoxyzolamide; GDC, glycine decarboxylase; GHA, glycine hydroxamate; PEPC, phosphoenolpyruvate carboxylase; TCA, tricarboxylic acid.

Introduction

Photosynthetic carbon fixation is a major component of plant productivity. During photosynthesis, Rubisco fixes CO_2 leading to the formation of end products such as sugars and starch. Compared with the levels of CO_2 in the atmosphere, the dissolved CO_2 within plant cells and available for chloroplasts is generally low and limiting. The diffusion of CO_2 in the stroma can be facilitated by carbonic anhydrase (CA) and increasing the availability of CO_2 for Rubisco in chloroplasts (Badger 2003, Badger and Pfanz 1995, Edwards and Walker 1983, Fett and Coleman 1994, Moroney et al. 2001, Villarejo et al. 2001).

The first evidence of involvement of CA in increasing the CO_2 affinity of CO_2 fixation by facilitating the CO_2 diffusion in chloroplasts was provided by Werdan and Heldt (1972). In the presence of CA, the carboxylation rate of Rubisco was enhanced by about 50%, while the O_2 consuming reaction was reduced to a similar extent (Okabe et al. 1980). The importance of CA in photosynthesis has been also suggested from the reduction in photosynthetic CO_2 fixation by inhibitors such as acetazolamide (AZA) or ethoxyzolamide (EZA) on the apparent photosynthesis of isolated chloroplasts and whole leaves (Badger 2003, Everson 1970, Jacobson et al. 1975, Sultemeyer et al. 1993). The deficiency of CA invariably resulted in a marked decrease in their capacity of CO_2 concentrating mechanism and subsequently photosynthesis of several algal mutants (Giordano et al. 2005, Hanson et al. 2003, Spalding et al. 2002, Thyssen et al. 2003). In algae (including diatoms), the levels of CO_2 can be increased by the help of CA. The role of CA and phosphoenolpyruvate carboxylase (PEPC) in CO_2 concentrating mechanism was demonstrated by the marked induction of these two enzymes when cells were grown at low CO_2 and sensitivity to EZA/AZA or 3,3-dichloro-2-dihydroxy phosphinoyl-methyl-2-propenoate (DCDP) (Badger 2003, Giordano et al. 2003, 2005, Hanson et al. 2003, Reinfelder et al. 2004).

Within green tissue, CO_2 is released during reactions of the tricarboxylic acid (TCA) cycle and/or photorespiration. In air at moderate temperature, the rate of photorespiratory CO_2 release is approximately 25% of the net rate of CO_2 assimilation (Sharkey 1988). The CO_2 released during photorespiration comes almost exclusively from glycine oxidation by glycine decarboxylase (GDC) (Husic et al. 1987). Aminoacetonitrile (AAN) and glycine hydroxamate (GHA) are inhibitors of GDC and their effects on photosynthesis are shown in wheat and tobacco leaves (Lawyer and Zelitch 1979, Usuda et al. 1980). Aminooxyacetate (AOA) is an inhibitor of serine:glyoxylate aminotransferase and is known to inhibit photosynthesis in mesophyll protoplasts of tobacco and leaf sections of barley (Jenkins et al. 1983, Rey and Peltier 1989). AOA can also inhibit nicotinamide adenine dinucleotide phosphate-dependent activity of glyoxylate reductase (Kleczkowski et al. 1987), and possibly pyridoxal phosphate-dependent enzymes.

Mesophyll protoplasts offer an attractive system to study not only photosynthesis but also several aspects of plant metabolism (Devi et al. 1992). During studies on photosynthesis by mesophyll protoplasts of pea, high rates of photosynthetic oxygen evolution were recorded, usually at 1 m*M* bicarbonate (ca. 74.1 μ*M* CO_2) (Padmasree and Raghavendra 1999a). This level is in strong contrast with the response of chloroplasts, which require up to 10 m*M* bicarbonate (ca. 588 μ*M* CO_2) for their photosynthesis (Edwards and Walker 1983). The reasons for such low CO_2 requirement of mesophyll protoplast photosynthesis are not clear. The markedly low requirement of CO_2 for photosynthesis by protoplasts of pea could be because of a CO_2 concentrating mechanism or high internal CO_2.

The present work is an attempt to study the influence of selected metabolic inhibitors (of CA or PEPC or TCA cycle or photorespiration) on photosynthesis by mesophyll protoplasts of pea. Experiments were conducted at conditions where photorespiratory metabolism is decreased, e.g. high CO_2 or low oxygen. The extent of photorespiratory decarboxylation at these conditions was assessed by determining glycine and serine, as the ratio of glycine/serine is known to increase when photorespiration is restricted.

Materials and methods

Plant material

Pea (*Pisum sativum* L. cv. Arkel) plants were raised from seeds (procured from Pocha Seeds Company Ltd., Pune, India) in green house with average temperatures of 25–30°C (day) and 20–25°C (night). The first and second fully expanded leaves were picked from 8- to 10-day-old plants and used for isolating mesophyll protoplasts.

Isolation of mesophyll protoplasts and chloroplasts

Mesophyll protoplasts were isolated from pea leaves by minor modifications of the procedure of Devi et al. (1992) by using Cellulase Onozuka R-10 and Macerozyme R-10 as described in detail, elsewhere (Padmasree and Raghavendra 1999a, Saradadevi and Raghavendra 1992). Chlorophyll was estimated by extraction into 80% acetone (Arnon 1949). Intact chloroplasts were prepared

by passing mesophyll protoplasts several times from a 2-ml disposable syringe through a 19-μm nylon filter. The chloroplasts were purified as described by Padmasree and Raghavendra (1999b).

Intactness of protoplasts and chloroplasts

The intactness of protoplasts, occasionally assessed by using the fluorescein diacetate uptake or glycolate oxidase assay, ranged from 90 to 95%. The intactness of chloroplasts assayed by using ferricyanide reduction (Walker 1988) ranged from 80 to 90%.

Monitoring photosynthesis: CO_2-dependent oxygen evolution

Photosynthetic oxygen evolution by mesophyll protoplasts or chloroplasts was monitored using a Clark type O_2 electrode (DW2, Hansatech Ltd., King's Lynn, UK). The 1-ml reaction medium for monitoring photosynthesis by mesophyll protoplasts contained 0.4 *M* sorbitol, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* Hepes-KOH, pH 7.5 and protoplasts equivalent to 10 μg Chl. The 1-ml reaction medium for the assay of photosynthesis by chloroplasts contained 0.4 *M* sorbitol, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 25 m*M* ethylenediaminetetraacetic acid, 150–200 U of catalase, 5 m*M* PPi, 0.9 m*M* adenosine triphosphate (ATP), 50 m*M* Hepes-KOH, pH 7.6 and chloroplasts equivalent to 10 μg Chl (Padmasree and Raghavendra 1999b). This assay is a modification of that described by Edwards et al. (1978). Sodium bicarbonate was added at required concentrations. The CO_2 levels were calculated using Henderson and Hasselbalch equation, taking into account the pH and bicarbonate concentration and assuming pKa of 6.37 for $NaHCO_3$ (Tsuzuki et al. 1985). The solutions were prepared in distilled water, bubbled with N_2. The reaction medium was bubbled with N_2 for 5 min before adding to the reaction chamber. The CO_2 concentration in these media was estimated and found to be <2 μ*M*.

Water at a constant temperature of 25°C was circulated through the outer jacket of the reaction chamber. A 35-mm slide projector provided the illumination of 600 μE m^{-2} s^{-1}. Calibration of the oxygen content in the electrode chamber was done with air-saturated water, assumed to contain 253 nmol O_2 ml^{-1} at 25°C (Walker 1988).

Lowering oxygen and test compounds

Prior to monitoring photosynthesis, the reaction medium was again bubbled with nitrogen gas to lower the O_2 concentration. The normal O_2 concentration in the medium was ca. 212 nmol ml^{-1}, while after bubbling with nitrogen the O_2 concentration decreased to ca. 65 nmol ml^{-1}. Protoplasts were pre-incubated in darkness for 10 min prior to illumination. The inhibitors were added to the reaction medium during pre-incubation to give the required final concentrations as indicated in the text.

AZA and or EZA were dissolved in DMSO. Control experiments included DMSO for comparison. AOA, AAN and GHA were dissolved in reaction medium and the pH was adjusted to 7.5 with either KOH (in case of AOA/AAN) or HCl (in case of GHA).

Amino acid analysis

Samples for amino acid analysis were prepared as described by Trethewey et al. (1998). The protoplast samples equivalent to 10 μg Chl were pelleted by centrifugation. To the pellet, 500 μl of 100% of ethanol and 500 μl of 80% ethanol were added successively. The samples were incubated at 70°C for 90 min and cleared by centrifugation (15 000 *g*, 10 min). After centrifugation, the supernatant was vacuum dried and dissolved in Na_2HPO_4 (pH 6.8) containing 0.4% tetrahydrofurane. Individual amino acids were analyzed after derivatization with *o*-phthalaldehyde, using a Hypersil 120 ODS column (Knauer, Berlin, Germany), as described by Hagemann et al. (2005).

Replications

The data presented are the average values of results (± standard error) from three to four experiments conducted on different days.

Results

CO_2-dependent O_2 evolution by mesophyll protoplasts and chloroplasts

The photosynthetic O_2 evolution by mesophyll protoplasts was stimulated by the presence of CO_2 and the rate of photosynthesis reached maximum at 74.1 μ*M* CO_2 (1 m*M* bicarbonate, pH 7.5) (Fig. 1A). The pattern of photosynthesis in intact chloroplasts of pea at different CO_2 concentrations was in strong contrast with the mesophyll cell protoplasts of pea. Maximum photosynthetic oxygen evolution by chloroplasts required 588 μ*M* CO_2 (10 m*M* bicarbonate, pH 7.6) (Fig. 1B).

Monitoring photosynthesis in the presence and absence of inhibitors

The sensitivity of photosynthesis by mesophyll protoplasts of pea to inhibitors of CAs or glycolysis or TCA cycle or

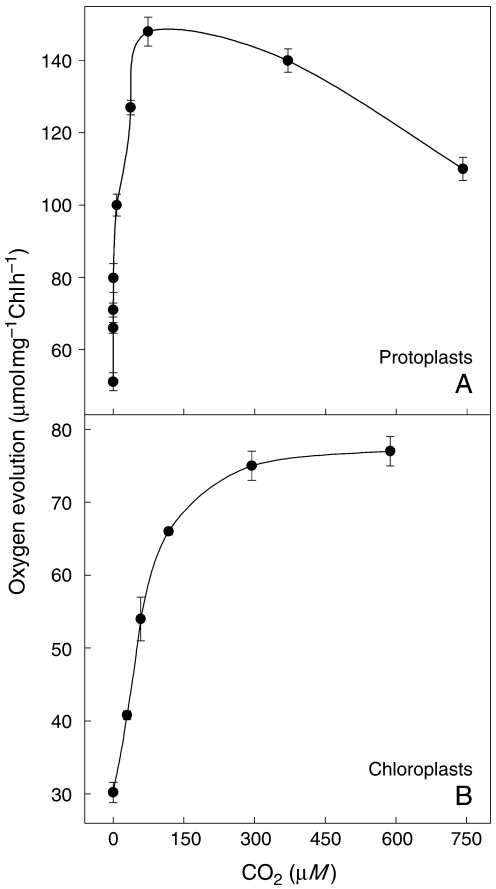

Fig. 1. Pattern of photosynthetic oxygen evolution by mesophyll protoplasts (A) and (B) intact chloroplasts of pea, in response to varying concentrations of CO_2 in the reaction medium. The inclusion of 100 Wilbur–Andersson units of carbonic anhydrase in the assay medium for chloroplasts did not alter their pattern of response. The use of Pi, instead of PPi + adenosine triphosphate, lowered slightly the photosynthetic rate but the pattern of response to CO_2 was similar.

photorespiratory cycle was evaluated. Further, the pattern of photosynthesis and sensitivity to these inhibitors was examined at either low (7.41 μ*M*) or high (741 μ*M*) CO_2. The presence of NaF (inhibitor of glycolysis) or sodium malonate (inhibitor of succinate dehydrogenase of TCA cycle) or DCDP (inhibitor of PEPC) did not have any significant effect on photosynthetic rate (Table 1).

Effect of inhibitors of CA and photorespiration

The CO_2 within plant cells can be increased by CA-mediated CO_2 concentrating mechanism and/or decarboxylating reactions of photorespiration. The effect of different inhibitors of CA or photorespiration was assessed at low (7.41 μ*M*) or high (741 μ*M*) CO_2. AZA, a CA inhibitor (Ignatova et al. 2001, Sultemeyer et al. 1993), had only a marginal effect on photosynthesis at low CO_2, with almost no effect at high CO_2 (Fig. 2). In contrast, photosynthesis was inhibited by >50% by EZA, inhibitor of principally internal CA (Ignatova et al. 2001, Sultemeyer et al. 1993) at low CO_2 (Fig. 3). Even at high CO_2, the inhibition of photosynthesis by EZA was about 15%. Our results on the effects of EZA on photosynthesis (Fig. 3) are quite similar to those of Ignatova et al. (2001).

Table 1. Effect of selected inhibitors on photosynthetic oxygen evolution by mesophyll protoplasts of pea. The inhibitors were 3,3-dichloro-2-dihydroxy phosphinoyl-methyl-2-propenoate (DCDP) (to inhibit phosphoenolpyruvate carboxylase) or NaF (to inhibit glycolysis) or malonate (to inhibit tricarboxylic acid cycle). Photosynthesis was monitored at low (7.41 μ*M*) or high (741 μ*M*) CO_2. Figures in parentheses represent the rates as percentages of the respective control without the test compound.

	CO_2 (μ*M*)	
Inhibitor	7.41	741
Photosynthetic rate (μmol mg^{-1} Chl h^{-1})		
None	95 ± 2.5 (100)	120 ± 1.0 (100)
DCDP (1 m*M*)	97 ± 3.5 (102)	115 ± 2.0 (105)
NaF (10 m*M*)	103 ± 1.0 (108)	112 ± 2.0 (107)
Malonate (10 m*M*)	101 ± 1.0 (106)	115 ± 2.5 (104)

The rates of photosynthetic oxygen evolution were suppressed markedly by inhibitors of photorespiratory metabolism. Photosynthesis was progressively suppressed as the concentration of GHA (inhibitor of GDC) increased. At 6 m*M* GHA, more than 50% inhibition of

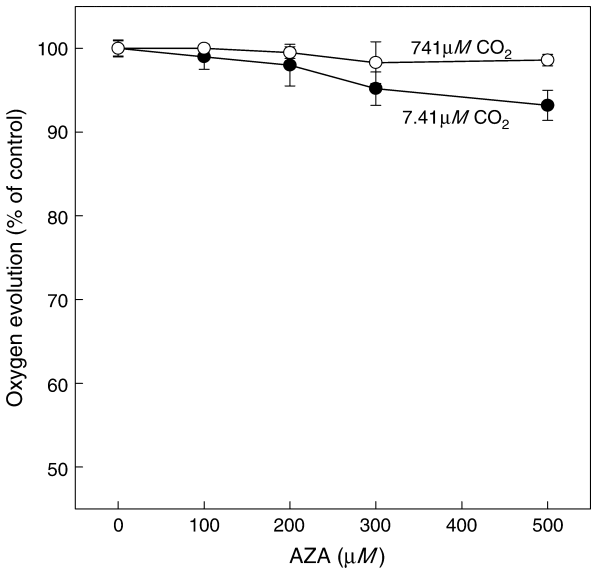

Fig. 2. Effect of acetazolamide (AZA) (inhibitor of carbonic anhydrase) on photosynthesis by mesophyll protoplasts of pea at low (7.41 μ*M*) or high (741 μ*M*) CO_2. The rate of photosynthesis in the absence of AZA, at low and high CO_2, was 92 and 124 μmol mg^{-1} Chl h^{-1}, respectively.

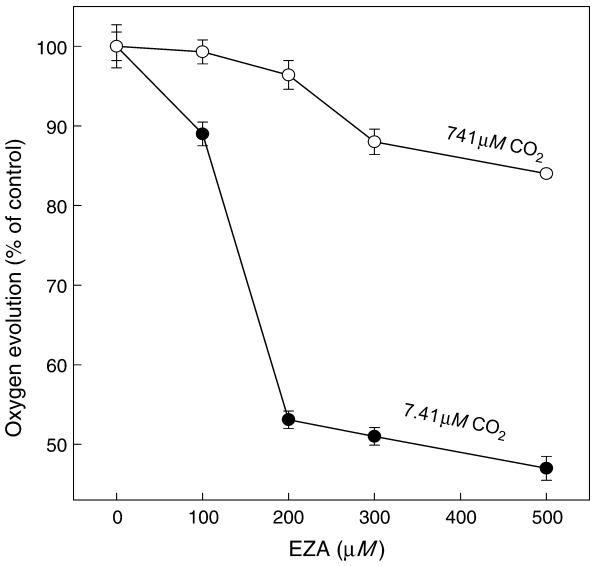

Fig. 3. Effect of ethoxyzolamide (EZA) (inhibitor of carbonic anhydrase) on photosynthesis by mesophyll protoplasts of pea at low (7.41 μ*M*) or high (741 μ*M*) CO_2. The rate of photosynthesis in the absence of EZA was at low and high CO_2, was 94 and 130 μmol mg^{-1} Chl h^{-1}, respectively.

photosynthesis was observed, at low CO_2 (Fig. 4). AAN (inhibitor of GDC) or AOA (inhibitor of serine: glyoxylate aminotransferase) suppressed photosynthesis at low CO_2 by 19–24% (Table 2). There was no inhibition of photosynthesis by AAN or AOA or GHA, at high CO_2.

The effects of 0.5 m*M* AZA, 0.5 m*M* EZA and 6 m*M* GHA on photosynthesis by isolated chloroplasts were

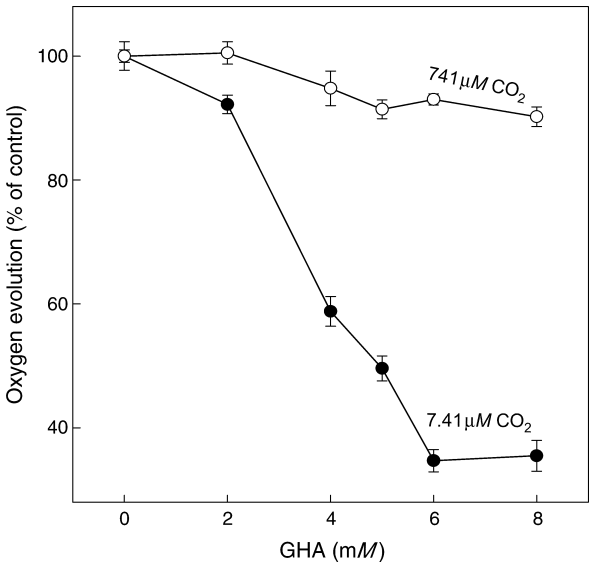

Fig. 4. Effect of glycine hydroxamate (GHA) (inhibitor of glycine decarboxylase) on photosynthesis by mesophyll protoplasts of pea at low (7.41 μ*M*) or high (741 μ*M*) CO_2. The rate of photosynthesis in the absence of GHA was 84 and 118 μmol mg^{-1} Chl h^{-1}.

Table 2. Effect of selected photorespiratory inhibitors on photosynthetic oxygen evolution by mesophyll protoplasts of pea. The inhibitors were aminoacetonitrile (AAN) [to inhibit glycine decarboxylase (GDC)] or glycine hydroxamate (GHA) (to inhibit GDC) or aminooxyacetate (AOA) (to inhibit serine: glyoxylate aminotransferase). Photosynthesis was monitored at low (7.41 μ*M*) or high (741 μ*M*) CO_2. Figures in parentheses represent the rates as percentages of the respective control without the test compound.

	CO_2 (μ*M*)	
Inhibitor	7.41	741
Photosynthetic rate (μmol mg^{-1} Chl h^{-1})		
None	106 ± 2.0 (100)	121 ± 0.6 (100)
AAN (5 m*M*)	80 ± 1.1 (76)	111 ± 1.5 (91)
GHA (6 m*M*)	66 ± 2.5 (62)	112 ± 1.2 (94)
AOA (5 m*M*)	85 ± 3.5 (81)	119 ± 1.0 (98)

studied. However, AZA, EZA or GHA had no significant effect and the decrease in photosynthesis ranged from 0 to 6% (Table 3).

Photosynthesis under low O_2 conditions

The extent of photorespiration can be reduced by lowering the O_2 concentration in the medium. Experiments were therefore conducted at low O_2. At low CO_2 (<37 μ*M*), the photosynthetic oxygen evolution under low oxygen was much less than that in normal O_2 (Fig. 5A). However, the photosynthetic rate continued to increase and the rate at 371 or 741 μ*M* CO_2 was more than that at normal O_2 (Fig. 5B). The decrease in photosynthesis by GHA was very high at normal O_2 but not at low O_2 (Fig. 6).

The levels of glycine and serine would reflect the operation of photorespiration. The ratio of glycine to serine in protoplasts was maximum at low 7.41 μ*M* CO_2 and normal O_2. The glycine/serine ratio was elevated markedly in GHA-treated samples at normal O_2. However, at low O_2, the glycine/serine ratio was not much affected by GHA (Table 4).

Table 3. Effect of selected inhibitors of carbonic anhydrase or photorespiration on photosynthetic oxygen evolution by isolated chloroplasts of pea at 588 μ*M* CO_2. Figures in parentheses represent the rates as percentages of control (without the inhibitors). Average of two experiments. AZA, acetazolamide; EZA, ethoxyzolamide; GHA, glycine hydroxamate.

Inhibitor	Photosynthetic rate (μmol mg^{-1} Chl h^{-1})
None	70 (100)
AZA (0.5 m*M*)	70 (100)
EZA (0.5 m*M*)	66 (94)
GHA (6 m*M*)	68 (97)

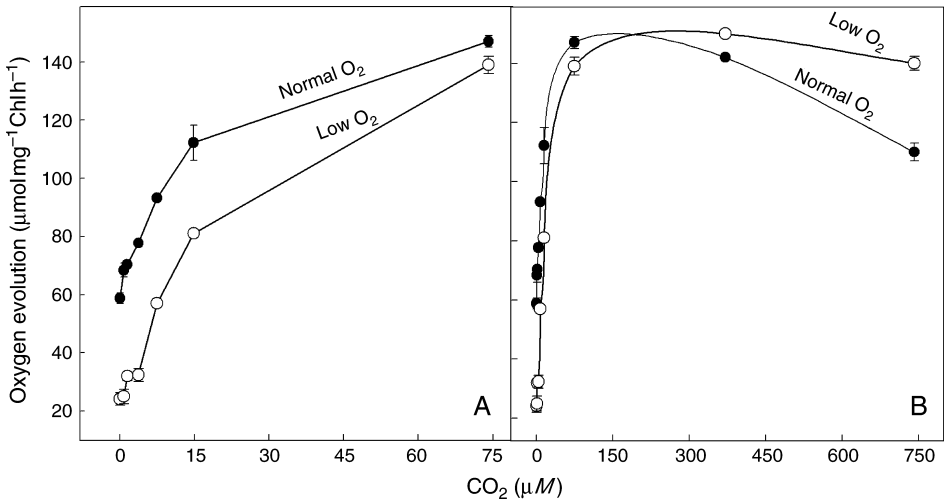

Fig. 5. Pattern of photosynthetic oxygen evolution by mesophyll protoplasts of pea in response to varying concentrations of CO_2, at either normal (212 nmol ml^{-1}) or low (65 nmol ml^{-1}) oxygen in reaction medium (B). Panel A represents the pattern of protoplast response at CO_2 concentrations of <75 μM.

Discussion

Low CO_2 requirement of protoplasts, compared with chloroplasts

Very few reports are available on the response of protoplast photosynthesis to varying concentrations of added CO_2. The present study establishes that photosynthesis by pea mesophyll protoplasts reaches its maximum rate at much less CO_2 levels than that required by chloroplasts (Fig. 1A, B). The requirement of 5–10 mM bicarbonate (ca. 294 to 588 μM CO_2 at pH 7.6) in case of chloroplast photosynthesis has been observed by others (Edwards et al. 1978, Walker et al. 1971). The response of photosynthesis by isolated chloroplasts to bicarbonate also depends on phosphate in the medium. Maximum rate was obtained at 588 μM CO_2 in the presence of optimum Pi by Usuda and Edwards (1982). In our experiments, photosynthesis by intact chloroplasts of pea with PPi and Pi reached maximum at 588 μM CO_2 (Fig. 1). The use of Pi instead of PPi + ATP resulted in a slight decrease in photosynthetic rate but chloroplasts still required 588 μM CO_2 for maximal rates (data not shown) indicating that Pi was either not limiting or not affecting CO_2 response.

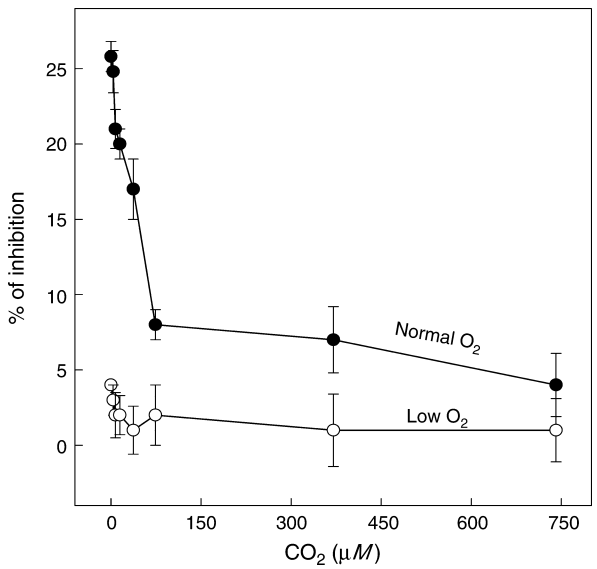

Fig. 6. Inhibition by 6 mM glycine hydroxamate (GHA) of photosynthetic oxygen evolution by mesophyll protoplasts of pea at varying concentrations of CO_2 and either normal or low O_2. The inhibition by GHA was calculated as % of respective control (in the absence of GHA). The typical pattern of photosynthesis in the absence of GHA, at low and normal O_2, is shown in Fig. 5.

Table 4. Levels of glycine (gly) and serine (ser) in protoplasts. Protoplasts were allowed to photosynthesize at either normal (212 nmol ml^{-1}) or low (65 nmol ml^{-1}) O_2. The protoplasts were supplied with either low (7.41 μM) or high (741 μM) CO_2. Results from a typical experiment. *Not done. GHA, glycine hydroxamate.

	No GHA			6 mM GHA		
	Gly	Ser	Gly/Ser	Gly	Ser	Gly/Ser
Condition	(μmol mg^{-1} Chl)		(Ratio)	(mmol mg^{-1} Chl)		(Ratio)
Normal O_2 and low CO_2	0.06	0.02	3.00	0.10	0.02	5.00
Low O_2 and low CO_2	0.08	0.03	2.66	0.07	0.02	3.50
Normal O_2 and high CO_2	0.05	0.02	2.50	ND*	ND	ND

Importance of CA at low CO_2

Active CO_2-concentrating mechanisms are known to operate in several algae, facilitated by CO_2 transporters across the membranes and activities of CA and PEPC within the cells (Badger and Price 2003, Badger et al. 2002, Geib et al. 1996, Kaplan and Reinhold 1999, Price et al. 1998). Different forms of CA exist in different subcellular locations, the plasma membrane, chloroplast, cytoplasm and even in mitochondria (Ignatova et al. 1998, Parisi et al. 2004, Perales et al. 2005, Reed and Graham 1981, Sultemeyer et al. 1993). The importance of CA in supporting photosynthetic CO_2 fixation has been demonstrated in chloroplasts and cellular systems by using typical inhibitors of CA such as AZA or EZA (Badger 2003, Everson 1970, Sultemeyer et al. 1993). The sensitivity of photosynthesis to EZA suggests that intrinsic CA may help to concentrate CO_2 in mesophyll protoplasts of pea. The higher sensitivity of photosynthesis to EZA at low CO_2 than that at high CO_2 endorses this suggestion (Fig. 3). Okabe et al. (1980) observed that the role of CA in CO_2 fixation is more pronounced at low external CO_2.

Possible role of photorespiratory CO_2

Within the cell, CO_2 can be released in the reactions of mitochondrial respiration and/or photorespiration, contributing to the pool of internal CO_2. However, the photosynthesis of mesophyll protoplasts was not significantly affected by either NaF or malonate, inhibitors of glycolysis and TCA cycle, respectively (Table 1), suggesting that TCA cycle may not be a significant source of internal CO_2. DCDP and malonate are powerful inhibitors of PEPC (Jenkins 1989, Raghavendra and Das 1975). Thus, the insensitivity of photosynthesis to DCDP implies that PEPC may not be a significant factor.

Photorespiratory metabolism is sensitive to AOA, GHA or AAN (Jenkins et al. 1983, Lawyer and Zelitch 1979, Rey and Peltier 1989). The marked suppression of photosynthesis by mesophyll protoplasts by AAN or AOA or GHA (Table 2) suggests that photorespiratory CO_2 is an important source of CO_2 within mesophyll protoplasts. The sensitivity of photosynthesis to these photorespiratory inhibitors (AAN or AOA or GHA) was higher at low CO_2 than at high CO_2 (Table 2). With increasing CO_2 concentration, the sensitivity of photosynthesis to GHA decreased (Fig. 6). This observation is in agreement with the results of Rey and Peltier (1989) obtained with mesophyll protoplasts of tobacco. Further, the magnitude of CO_2 release from photorespiration is much higher than that from dark respiration, i.e. TCA cycle (Loreto et al. 1999).

Photorespiration can be modulated by changing the ambient O_2 concentration. The O_2 level in the reaction medium could be lowered by bubbling with nitrogen. At low O_2, the CO_2 requirement for photosynthesis increased, confirming that the availability of photorespiratory CO_2 was restricted at low O_2 (Fig. 5A, B). The extent of inhibition of photosynthesis by photorespiratory inhibitors was high at low CO_2 (Fig. 4). In contrast, photosynthesis was not much sensitive to GHA at low O_2 (Fig. 6). Obviously, photorespiration was decreased at low O_2, and the sensitivity of photosynthesis to GHA also was low.

When photorespiratory metabolism is restricted, as in mutants deficient in glutamine synthetase or in presence of GDC inhibitors, there is usually an increase in glycine/serine ratio, reflecting the accumulation of glycine (Bauwe and Kolukisaoglu 2003). In our experiments (Table 4), the presence of GHA (inhibitor of GDC) elevated markedly the ratio of glycine/serine (particularly at normal O_2) confirming that the glycine decarboxylation is decreased. However, the ability of GHA to elevate the glycine/serine ratio was much subdued at low O_2, indicating that under non-photorespiratory conditions, GHA was not as effective as at normal O_2 or active photorespiratory conditions. These observations confirm that the photorespiratory decarboxylations are reduced at low O_2 and in presence of GHA.

Photorespiratory CO_2 and CA may help in photosynthetic carbon assimilation

A significant level of photorespiratory CO_2 recycling during photosynthesis is frequently suggested. However, the exact determination of the rates of photorespiratory CO_2 evolution and O_2 uptake is difficult. Varied estimates of photorespiration have been made by different methods (Catzky et al. 1971, Jackson and Volk 1970). More than 80% of the photorespiratory CO_2 is recycled inside the leaf in ambient CO_2 concentration and substantial recycling is known to occur in a number of plant species (Loreto et al. 1999). Recycling of carbon was assessed by measurements of CO_2 incorporation (Ludwig and Canvin 1971) and by theoretical estimations (Gerbaud and Andre 1987, Peisker 1980). Some of the results indicated that up to 35% of photorespiratory CO_2 is refixed (Bravdo 1968, Ludwig and Canvin 1971, Samish and Koller 1968). Our experiments suggest that photorespiration can be an important source of internal CO_2 for carbon assimilation in mesophyll cells.

An ability to use exogenous HCO_3^- as well as CO_2 as a carbon source for photosynthesis, suggests function of CA during the operation of the carbon concentrating mechanism (CCM) (Sultemeyer et al. 1993). Cyanobacteria have evolved an extremely effective single-cell CCM (Badger and Price 2003). In C_4 and CAM higher plants, cytosolic CA plays a vital role in the conversion of CO_2 to

HCO_3^-, which is a substrate for PEP carboxylase. In case of C_3 plants CA facilitates diffusion of CO_2 through the cytosol and the chloroplasts stroma. Rubisco and CA association is a common feature of active CCMs (Badger and Price 1994).

Recycling of respiratory CO_2 in CAM photosynthesis is known to be significant and important (Cushman 2001). Limited recycling of photorespiratory CO_2 during photosynthetic carbon fixation was suggested in C_3 species, such as *Brassica oleracea*, *Cichorium intybus*, *Spinacia oleracea*, *Vigna unguiculata*, *Helianthus annus* (Loreto et al. 1999). It is very tempting to speculate that photorespiratory CO_2 can be a significant source for carbon assimilation in mesophyll protoplast. We propose that recycling of CO_2 can be an additional factor involved in the beneficial interactions of mitochondria, peroxisomes and chloroplasts, a concept now well established (Raghavendra and Padmasree 2003).

Apparent anomaly with earlier observations and other possible explanations

The results from our study appear to pose an anomaly to earlier observations. Tsuzuki et al. (1985) observed that the affinity of chloroplasts for CO_2 is higher than that of protoplasts in wheat. Their experiments on chloroplasts were conducted in presence of 50 m*M* DHAP. Because we did not include such high concentration of DHAP these experiments cannot be compared directly. The extensive work of Ogren (1984) established that the photosynthesis of plant cells is higher at low O_2 than at atmospheric levels of O_2. Although the data of Fig. 5A appear to be at variance, with this well-known concept, the data in Fig. 5B demonstrate that the photosynthesis of protoplasts is indeed higher in low O_2 than that in normal O_2.

There could be other explanations for the interesting difference in the carbon requirement of chloroplasts and protoplasts. Tsuzuki et al. (1985) reported that the apparent Km CO_2 of protoplasts was two-fold greater than that of chloroplasts and suggested that possible reasons were the high diffusive resistance for CO_2 and activation state of Rubisco in chloroplasts. The high CO_2 requirement of chloroplasts could be also because of slowness of photosynthetic induction and Rubisco activation (Edwards et al. 1978), mediated CO_2 transport mechanisms (Volokita et al. 1981) or simply because of the fact that these organelles are isolated.

Concluding remarks

Our results demonstrate that, at normal O_2, photorespiratory CO_2 can contribute significantly to the internal pool of CO_2 within mesophyll protoplasts. The internal CA may have a role in optimizing the availability of internal CO_2. The recent observations that CA is present in mitochondria (Parisi et al. 2004, Perales et al. 2005) further highlight the possible dynamic role of CA in higher plants. This article points out at the physiological importance of photorespiratory CO_2 and CA for photosynthesis in mesophyll cells. Further experiments are necessary to calculate precisely the extent of recycling of photorespiratory CO_2. CA would be an important component to concentrate or retain such CO_2.

Another interesting observation is the consistent decrease in photosynthesis of mesophyll protoplasts at high CO_2, e.g. 741 μM (Fig. 1A), unlike that of chloroplasts (Fig. 1B). This appears similar to the down-regulation of photosynthesis in leaves during prolonged exposure to high CO_2 (Amthor 1995). The marked inhibition of photosynthesis at high CO_2 in pea mesophyll protoplasts was studied extensively by the Russian group (Ignatova et al. 1993, 1999). The prevention by AZA of photosynthesis inhibition at high CO_2 suggested that the plasmalemma CA may play an important role in such inhibition at high CO_2.

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