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CONSUMPTION OF CARBONYL SULPHIDE (COS) BY HIGHER PLANT CARBONIC ANHYDRASE (CA)

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Abstract—Carbonic anhydrase (CA), isolated from pea leaves, was found to consume carbonyl sulphide (COS), a climatic relevant trace gas in the atmosphere. The isolated enzyme, free of other carboxylases, showed a very high affinity towards this substrate. The experiments confirm that CA is the key enzyme for the consumption of COS in higher plants. Furthermore, the identification of this enzyme furthers our understanding of additional sinks for COS, which are needed to understand the balance of known global sources. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

Reduced sulphur compounds, hydrogen sulphide (H_2S), methyl mercaptan (CH_3SH), dimethyl sulphide (CH_3SCH_3, DMS), carbon disulphide (CS_2) and carbonyl sulphide (COS) are exchanged between biosphere and atmosphere. Estimates of the natural sulphur emissions range between 35 and 70 Tg(S) a^{-1} (Andreae and Jaeschke, 1992), which is in the same order of magnitude as the man-made sulphur emissions (70–100 Tg(S) a^{-1}, Cullis and Hirschler, 1980). However, these estimates remain considerably uncertain (Kesselmeier, 1991). A considerable amount of sulphur reduced sulfur is deposited as COS (for review see Chin and Davis, 1993), which is the most abundant natural trace gas in the remote troposphere with an average concentration near 500 pptv (Torres *et al.*, 1980; Rasmussen *et al.*, 1982a; Khalil, 1992). The average concentration of COS is remarkably constant both vertically and latitudinally, though a general increase is observed in the northern hemisphere (Bingemer *et al.*, 1990). This constant steady state proves a well-balanced sink/source relation and/or a long atmospheric lifetime.

COS has the potential for cooling the earth. Because of its low tropospheric reactivity, it diffuses into the stratosphere and sustains the stratospheric sulphate layer (Crutzen, 1976) which affects the Earth's radiation balance and climate. Therefore, it is of interest to investigate how COS exchange is coupled to the biosphere. Besides emissions of COS by soil/plant systems (Aneja *et al.*, 1979a, b; Adams *et al.*, 1981; Steudler and Peterson, 1984, 1985; Carroll *et al.*, 1986) and higher plants (Lamb *et al.*, 1987; Rennenberg *et al.*, 1990; Berresheim and Vulcan 1992; Kesselmeier *et al.*, 1993a), previous studies indicate that COS can be taken up and metabolised by plants (Taylor *et al.*, 1983; Brown and Bell, 1986; Brown *et al.*, 1986; Goldan *et al.*, 1987, 1988; Fall *et al.*, 1988; Hofmann *et al.*, 1992a; Bartell *et al.*, 1993; Kesselmeier and Merck, 1993). The direction of the exchange is influenced by a COS compensation point as described by Kesselmeier and Merck (1993) and Kesselmeier *et al.* (1993b).

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As recently demonstrated by Protoschill-Krebs and Kesselmeier (1992), the carboxylating enzymes ribulose-1,5-bisphosphate-carboxylase (Rubisco) and phosphoenolpyruvate-carboxylase (PEP-Co) are able to consume COS by hydrolysis of COS to CO_2 and H_2S in addition to the pathway discussed by Lorimer and Pierce (1989), who showed that COS may serve as a direct substrate for Rubisco. However, the resulting product is of questionable significance for the physiology of plants. Furthermore, Protoschill-Krebs and Kesselmeier (1992) showed that the consumption of COS by the carboxylating enzymes was significantly enhanced by adding carbonic anhydrase (CA). Based on these investigations we discussed carbonic anhydrase (CA) as the key enzyme for the uptake of COS by higher plants. In close analogy to the CO_2 to HCO_3^- interconversion, we expected CA to be able to split COS into H_2S and CO_2. Investigations with rat hepatocytes and erythrocytes (Chengelis and Neal, 1979, 1980) and with cyanobacteria (Miller *et al.*, 1989; Badger and Price, 1990) gave reason to believe that COS is directly consumed by CA, because CA-inhibitors stop COS consumption or H_2S production, respectively. Also, as recently reported the green alga *Chlamydomonas reinhardtii* is able to consume COS in close relation to its amount of extracellular CA activity (Protoschill-Krebs *et al.*, 1995). However, experimental evidence for the consumption of COS by higher plant CA is lacking. Therefore, we investigated whether higher plant CA is capable of consuming COS. We isolated carbonic anhydrase from pea leaves and investigated the consumption of COS as well as this enzyme's affinity towards COS in comparison to CO_2.

MATERIALS AND METHODS

Plant material and cultivation

Seedlings of *Pisum sativum* were grown in hydroponic culture in a growth chamber under a day/night cycle of 12 h. The plants were illuminated with 302 μmol photons $m^{-2} s^{-1}$ (photosynthetic active radiation, PAR) at 23°C and 70% relative humidity. During darkness the temperature was lowered to 18°C at a relative humidity of 75%.

Isolation and purification of carbonic anhydrase (CA)

All stages of enzyme preparation were performed at 0–4°C. Leaves harvested from 3-week old peas (fresh weight 70–80 g) were homogenised in a cooled mixer, modified according to Kannangara *et al.* (1977), using the following blending medium: 150 mM K_2HPO_4, 1 mM dithiothreitol (DTT), 5% polyclar (w/v). The homogenate was filtered sequentially through nets of nylon with a pore size of 200 and 60 μm and centrifuged for 30 min at 27,000 × **g**. The supernatant was saturated with solid ammonium sulfate to 30% and centrifuged for 20 min at 27,000 × **g**. Within 30 min the supernatant was brought to 60% saturation with solid ammonium sulfate. After 20 min of centrifugation at 27,000 × **g** the precipitate obtained was dissolved in a small volume of extraction buffer. The suspension was further centrifuged for 15 minutes at 90,000 × **g**. The volume of the supernatant was diminished by ultrafiltration using centrifugal ultrafilters (Millipore, catalog number UFC2TTK02). The concentrate was applied to a Hiload 16/60 Superdex 75 column (Pharmacia catalog number 17-1068-01) equilibrated with extraction buffer. At a flow rate of 0.5 ml min^{-1}, CA was eluted after 70 ml as a single peak of activity. The active fractions were combined and precipitated with 60% ammonium sulfate as previously described. The precipitate was dissolved in a small volume of 150 mM KH_2PO_4, 5 mM dithiothreitol (DTT) and 60% $(NH_4)_2SO_4$. The ampules containing CA were flushed with argon, stoppered and stored at 4°C.

Protein determination

The protein content was determined according to Smith *et al.* (1985) by a complexation of bicinchonic acid (BCA–Protein Assay Reagent, Pierce, Germany, catalog number 23225) with proteins delivering a specific compound which was measured by photometry.

Dynamic headspace system

The COS concentrations in the assays were determined by means of the "dynamic headspace method" according to Rasmussen *et al.* (1982b) and Ferek and Andreae (1983). The method was modified to meet the needs of enzymatic investigations. Helium prepurified by passing it through a cryogenic trap submerged in liquid nitrogen, was constantly bubbled (30 ml min^{-1}) through a glass cylinder filled with 5 ml of a 20% citric acid solution in water. Aliquots of 100 μl were injected via a septum into the cylinder. Due to the acidity of the solution all enzymatic processes were stopped. Gases were transferred to a trapping unit, consisting of a Nafion drier (Permapure, MD 125, 1.8 m) and a cryogenic trap submerged in liquid nitrogen. For the trap construction and the reliability of the Nafion drier see Hofmann *et al.* (1992b).

GC technique and calibration

The GC analysis of the COS and the produced H_2S followed the method described by Hofmann *et al.* (1992b). Calibration was achieved by using a permeation device. The COS was diluted with pressurised N_2, which was prepurified by passing through a cryogenic trap submerged in liquid nitrogen. Aliquots of this mixture were either directly transferred to the GC with gas-tight syringes or injected into the dynamic headspace apparatus. This comparison of a direct injection of COS with an injection of calibration samples trapped after passing the dynamic headspace system filled with 20% citric acid showed only minor losses of COS (2%) within the headspace apparatus. Calibration of H_2S with these methods showed significant, though constant, losses of H_2S after passing the headspace system. Therefore, only the COS data were used for further evaluations.

Generation and calibration of COS solutions

A 25 mM sodium barbital–HCl (pH 8.2) was continuously bubbled with commercially available 0.01% COS (Alphagaz) in a tempered glass vessel at 20°C for at least 40 min. The concentration was measured by the dynamic headspace method described above. Concentrations were found to be 2.36 μmol COS ℓ^{-1} ($\pm$ 0.039; $n = 12$), comparable to the solubility of COS in water at 2.48 μmol ℓ^{-1} (Gmelin, 1977a). After transfer of the solution to ampules stoppered with gas-tight TEFLON coated septa, we measured 2.22 μmol ℓ^{-1} ($\pm$ 0.054; $n = 13$) after an incubation time of up to 20 min tested. Thus, the total loss was around 6% and hence lower or in the range of the non-enzymatic hydrolysis.

Enzyme assays

CO_2 assay of CA: CA was assayed by a modification of the electrometric method of Wilbur and Anderson (1948) using 25 mM sodium barbital–HCl (pH 8.2) including 5 mM DTT as assay buffer. The enzyme extract or test solution was equilibrated for at least 2 min in 4 ml of assay buffer. The

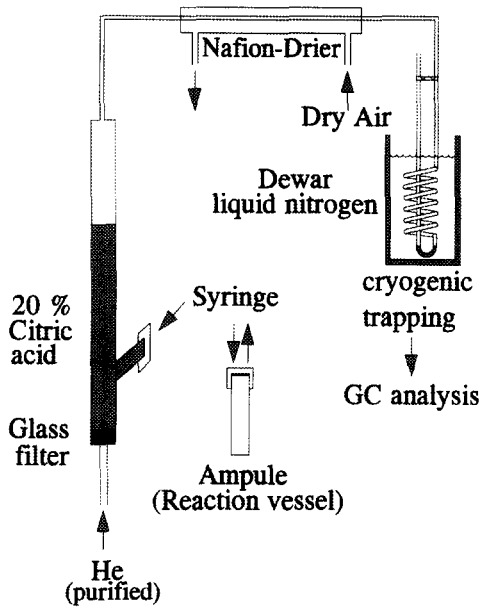

Fig. 1. Schematic overview: measurements of an enzymatically driven COS consumption with a modified dynamic headspace apparatus. For more explanation see Materials and Methods.

reaction was initiated by the addition of 1 ml of CO_2-saturated water (approximately 76 mM at 0°C, Gmelin, (1977b)). The time for the pH to decline from 8.2 to 7.7 was recorded. Enzyme units were calculated according to Porter and Grodzinski (1983).

COS *assay of CA*: Isolated carbonic anhydrase samples were injected into the COS solutions prepared as described above and filled into gas-tight ampules (vol. 1.85 ml). Incubation at 20°C was stopped by injecting aliquots into the degassing dynamic headspace system. Analysis of the COS content of the solutions were undertaken up to 20 min for the control and up to 6 min for the enzyme assay. A schematic overview is given in Fig. 1.

RESULTS AND DISCUSSION

Enzyme activities and properties

Table 1 shows the properties of the carbonic anhydrase in the different fractions obtained during isolation and purification. The isolated enzyme fraction obtained as a single peak after the gel filtration (see Materials and Methods) and used for the further studies showed a 21-fold enrichment as compared to the leaf extract fraction we started with. Ribulose-1,5-bisphosphate carboxylase (Rubisco) and phosphoenolpyruvate carboxylase (PEP-Co) activities tested according to Di Marco and Tricoli (1983) or Uedan and Sugiyama (1976), respectively, could not be detected in these fractions.

As already mentioned above (Materials and Methods), the calibration of H_2S showed significant though constant losses of H_2S between 30 and 40% after passing the headspace system. Therefore, only the COS data were used for further evaluations as these

Table 1. Enrichment of carbonic anhydrase during isolation

	Activity (units)	Protein (mg)	Specific activity (units mg^{-1})
Leaf extract	96,500	290	333
60% $(NH_4)_2SO_4$	106,000	175	606
Ultrafiltration[a]	91,500	117	782
Gelfiltration[b]	73,000	10.5	6952

[a] Millipore centrifugal ultrafilters.
[b] Hiload 16/60 Superdex 75 column (Pharmacia).

give a more reliable information. All our tests for the consumption of COS were carried out at pH 8.2, a pH value typical for the chloroplastic stroma under illumination. Although alkaline pH accelerates hydrolysis, COS is only slowly hydrolysed by strongly alkaline reagents such as sodium hydroxide (Ferm, 1957). Under these conditions the carbonic anhydrase (CA) isolated from pea leaves was able to accelerate the consumption of COS. As shown in Fig. 2a, CA enhanced the decrease of COS significantly as compared to the chemical hydrolysis. Starting with 130–135 ng COS per ml buffer (2.16–2.25 μM), the COS concentration decreases to 70 ng ml^{-1} within 6 min; without the presence of the enzyme the concentration has only slightly decreased even after 20 min. The CA-driven COS consumption increases linearly with the applied enzyme units as shown in Fig. 2b. Enzymatic tests under different pH ranges revealed no pH optimum but a steadily increasing COS consumption from pH 6 to pH 10 tested (data not shown). The expected denaturation of the enzyme under a pH higher than 8 seemed to be counterbalanced by the chemical hydrolysis of COS. However, the pH inside the plant chloroplasts does not reach such high pH values. Thus, the physiologically based consumption of COS is assumed to be of major importance under natural conditions.

Using different substrate (COS) concentrations, we estimated the Michaelis–Menten constant (K_M, Fig. 2c), representing the affinity of an enzyme towards its substrate (see Karlson, 1988). Thus, the K_M value for the consumption of COS by CA was found to be 39 μM. For comparison, the K_M value for the consumption of CO_2 by CA in the same plants is found at 34 mM (Lamb, 1977; Poincelot, 1979). Thus, the affinity of CA towards COS is a thousand times higher than for CO_2. This is of special importance with regard to the natural atmospheric mixing ratios of CO_2 (ppm range) and COS (pptv–ppbv range). The high affinity of CA for COS meets the requirements for the assumption that the carboxylating enzymes, including CA, are responsible for the uptake of COS by higher plants. Furthermore, such a high affinity may explain why plants exposed to various CO_2/COS mixtures prefer COS over CO_2 as found by comparing the CO_2/COS uptake ratios with atmospheric ratios (Kesselmeier and Merck, 1993).

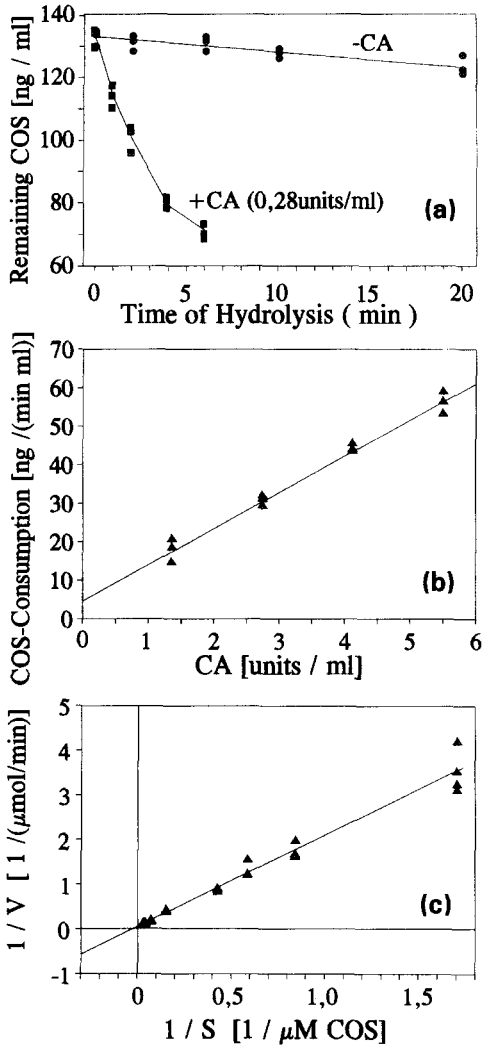

Fig. 2. COS consumption of carbonic anhydrase (CA) isolated from pea leaves. (A) Decrease of COS in samples incubated with or without CA. (B) COS consumption of CA and dependence on the applied enzyme units. (C) Lineweaver–Burk plot for the determination of the Michaelis Menten constant K_M by plotting the reciprocal values of the substrate concentration vs the consumption velocity. According to this procedure the K_M is found at the intersect of the regression line with the x-axis and thus is given as $-1/K_M$.

CONCLUSIONS

Based on the experiments described above, carbonic anhydrase can be regarded as the key enzyme for the COS metabolism in plants. Based on the high affinity of CA towards COS we conclude that carbonic anhydrase *in vivo* splits COS into CO_2 and H_2S and may feed the carboxylating enzymes ribulose-1,5-bisphosphate carboxylase (Rubisco) and phosphoenolpyruvate carboxylase (PEP-Co). This is in accordance with the recently published experiments (Protoschill-Krebs and Kesselmeier, 1992) showing that the carboxylating enzymes PEP-Co and Rubisco accelerated COS hydrolysis by the fixation of HCO_3^- and CO_2. In the presence of CA, the CO_2 fixation rate of both carboxylating enzymes was significantly increased. This means all enzymes involved in CO_2 assimilation may participate in the COS metabolism. Additionally, this metabolism can explain the H_2S emission in close relation to the COS deposition sometimes observed in field experiments (Bartell *et al.*, 1993). Furthermore, the high affinity for the substrate COS explains how CA is able to overcome the high concentration differences of COS (550 pptv) and CO_2 (350 ppmv) in the atmosphere. In addition, CA is known to be the enzyme with the highest molar activity (Poincelot, 1979) and to be able to saturate 100-fold the CO_2 demand of the Rubisco. Thus, as CA activity seems to be high enough, we may assume that the COS supply rather than the CA content and activity is the limiting factor for the COS uptake. However, as shown by Protoschill-Krebs *et al.* (1995), the CA-dependent COS consumption by the green alga *Chlamydomonas reinhardtii* can be significantly decreased by a 100-fold increase of the CO_2 concentration in the medium, due to an induction process, decreasing the amount of extracellular CA. If this reaction could also be awaited for higher plants, we might speculate about a decrease of COS consumption by vegetation under increasing atmospheric CO_2 concentrations.

The discussion of carbonic anhydrase as the key enzyme for the biologically based consumption of COS from the atmosphere by higher plants furthers the understanding of COS sinks. This pathway explains the consumption of COS by an irreversible cleavage into H_2S and CO_2. The further use of the products is not necessary but may occur. Furthermore, CA-induced uptake of COS may occur independently from light. Thus, sink calculations on a net primary production basis (CO_2 consumption) as frequently performed (Chin and Davis, 1993; Kesselmeier and Merk, 1993) are only reasonable if we assume a strict light-dependent stomatal control for the exchange of COS between higher (vascular) plants and the atmosphere. For organisms without a cuticle and without stomata or with light-independent stomata regulation, we also can expect the uptake and cleavage of COS in the dark and thus without any relation to the uptake of CO_2. As shown recently by Gries *et al.* (1994), lichens may be seen as potential consumers of COS from the atmosphere. These organisms were shown to consume COS independently from light. The uptake depended only on the water status of the lichens. Such an uptake can only be explained by a light-independent step in the consumption of COS. Carbonic anhydrase is a light-independent enzyme and is found in nearly all organisms. Thus, a CA-induced consumption of COS can explain this uptake and can open the discussion about other COS sinks (Kesselmeier *et al.*, 1996).

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