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PLURALITY OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN *SPINACEA OLERACEA*: SUBCELLULAR DISTRIBUTION, PARTIAL PURIFICATION, AND PROPERTIES

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Key Word Index—*Spinacea oleracea*; Chenopodiaceae; spinach leaf chloroplasts; cyclic nucleotide phosphodiesterase; cyclic nucleotides; subcellular fractionation.

Abstract—An active cyclic nucleotide phosphodiesterase has been partially purified from the 100000 *g* supernatant of a spinach homogenate. It precipitated at 20–40% saturation with $(NH_4)_2SO_4$ and was separated on a column of Sephadex G-200 into two major peaks of activity (peaks 1 and 2). Peak 1 (MW 5×10^5) was resolved by column chromatography on DEAE-cellulose into 5 protein fractions; two of these (1_c and 1_m) exhibited cyclic nucleotide phosphodiesterase activity. Subcellular fractionation showed that the phosphodiesterase of highest specific activity is located in the peroxisomes but that an enzyme of relatively high specific activity also occurs in the chloroplast and Golgi fractions. The largest total activity was in the microsomes. Isoelectric focussing of chloroplast phosphodiesterase activity gave two bands corresponding to peaks 1_c and 2. Similar examination of the microsomal, peroxisomal and Golgi fractions showed phosphodiesterases corresponding to peaks 1_m and 2. Peak 1_c activity is greater towards purine 3′,5′-cyclic nucleotides than towards their 2′,3′-isomers; the converse is true of peak 1_m. Examination of the properties of 1_c and 1_m showed a number of other differences. The pH optimum of 1_c is 6.1 and that of 1_m is 4.9. Theophylline (0.1 mM) inhibited 1_c to a greater extent than it did 1_m; Ca^{2+} stimulated 1_c activity but had no effect on 1_m. Pre-incubation with trypsin inhibited 1_m activity whereas similar treatment of 1_c gave an initial 5-fold stimulation. Repeated freezing and thawing of preparations 1_c and 1_m also evoked a difference in response. These results were shown to be attributable to removal of an inhibitor from 1_c. Evidence is presented that an endogenous activator is also present.

INTRODUCTION

Despite criticism [1, 2], early reports of the isolation of 3′:5′-cyclic AMP from tissues of higher plants [3, 4] have recently been further substantiated [5, 6]. A possible physiological role of cyclic AMP in higher plants, however, remains controversial. It has been argued [7] that the overall concentration of cyclic AMP in plants is too low to be indicative of a physiological function, especially in connexion with the mediation of phytohormone effects. This argument ignores the possibility that localization of cyclic AMP, and its related enzymes, in specific species of subcellular organelles could result in regulated local concentrations of physiological significance. With this in mind, the present work sought to examine the subcellular distribution in plant tissues of cyclic nucleotide phosphodiesterase. Spinach was used, rather than *Phaseolus* as in our previous studies, because of the relative ease with which it can be fractionated. Advantage was taken of the subsequent availability of spinach phosphodiesterase in order to examine its properties. Cyclic nucleotide phosphodiesterase activity has been demonstrated in extracts from a number of plant tissues [8–13]. Some authors suggest, however, that plant phosphodiesterases differ substantially from those obtained from bacterial and animal sources and that they are primarily concerned with catabolism of 2′:3′-cyclic AMP during enzymic degradation of RNA. Our previous studies with *Phaseolus* [12, 13] revealed the presence of a phosphodiesterase with properties more like those of the animal and bacterial systems concerned in the metabolism of 3′:5′-cyclic AMP. An additional objective of the present work was to test the implication that more than one type of cyclic nucleotide phosphodiesterase is present in plant tissues.

RESULTS

After incubation of 3′:5′-cyclic AMP-[8-^{3}H] with the 100000 *g* supernatant from a spinach homogenate, the radioactive products were identified by TLC as 3′-AMP, 5′-AMP and adenosine; all three compounds were absent from the control radiochromatogram. An unidentified radioactive product was also observed to be present in the incubate. This has a similar R_f value to that of an authentic sample of cyclic AMP but was not a contaminant of the cyclic AMP-[8-^{3}H] used as substrate. Production of 3′- and 5′-AMP from cyclic AMP in the incubate and their absence from the control indicates the presence of an active cyclic nucleotide phosphodiesterase in the supernatant. Concomitant production of radioactive adenosine is attributable to nucleotidase activity in the preparation, but as both 3′- and 5′-AMP were also present in the incubate, it is concluded that the nucleotidase is less active than the phosphodiesterase. After addition of 3′- and 5′-nucleotidase, the nucleoside monophosphates remaining in the reaction mixture were completely hydrolysed to adenosine and Pi, so enabling the latter to be used as a quantitative indicator of phosphodiesterase activity.

Examination of the fractions obtained from the supernatant by fractional precipitation with $(NH_4)_2SO_4$,

Table 1. Partial purification of cyclic AMP phosphodiesterase from *Spinacea oleracea*

Fraction	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Fold purification	Recovery of activity (%)
100000 *g* supernatant	21.3	0.02	1.0	100
100000 *g* supernatant after dialysis	135.7	0.21	10.5	637
20–40% sat. $(NH_4)_2SO_4$	65.6	0.66	33.0	307
Sephadex G-200				
Peak 1	24.0	3.0	150.0	112.6
Peak 2	17.0	3.4	170.0	79.8
DEAE-cellulose				
peal 1_c	7.0	5.3	265.0	32.8
peak 1_m	10.0	5.0	250.0	46.9

showed that 86.5% of the phosphodiesterase activity was located in the 20–40% saturated fraction. Gel-filtration chromatography of this fraction on Sephadex G-200, resolved it into two major peaks, eluting at 50 and 200 ml; cyclic nucleotide phosphodiesterase was present in both. The protein in peak 1 had an apparent MW of 5×10^5, and that in peak 2 had a MW of 3.7×10^4. The activity of peak 1 was further fractionated by chromatography on a column of DEAE-cellulose. Five protein peaks were obtained; two of them contained cyclic nucleotide phosphodiesterase activity. Of the two peaks of activity, one (peak 1_c) eluted in the buffer used to wash the column, and the other (peak 1_m) eluted in 0.375 M NaCl. Isoelectric focussing of peaks 1_c, 1_m and 2 showed their isoelectric points to be 6.3, 4.7 and 3.7, respectively.

Details of the purification of the cyclic nucleotide phosphodiesterases are given in Table 1. A 265-fold purification of the high MW activity (peak 1_c) was obtained; recovery was 32.8%. The other high MW activity (peak 1_m) was purified 250-fold with a recovery of 46.9%. The low MW activity (peak 2) was purified 170-fold by the procedure which culminated in gel-filtration; a recovery of 79.8% was obtained. Anion-exchange chromatography on DEAE-cellulose produced too low a recovery (<5%) of peak 2 to be of practical value.

Dialysis of the 100000 *g* supernatant (Table 1) resulted in a substantial increase in activity. Addition of 1 mM NaH_2PO_4 lowered this activity to its pre-dialysis level.

Table 2 shows the distribution of cyclic nucleotide phosphodiesterase activity in various subcellular fractions of spinach leaves. Fractionation was effected by zonal centrifugration as described in Experimental. One protein band (density 1.22 g/ml) obtained during the fractionation was devoid of phosphodiesterase activity. This band was identified as the mitochondrial fraction by its cytochrome oxidase activity (1.25 μmol/min/mg protein) and presence of an Antimycin-sensitive NADH cytochrome *c* reductase activity. Each of the other fractions exhibited phosphodiesterase activity. As determined by measuring the activity of marker enzymes, the purity of each of the subcellular fractions was *ca* 90%.

The phosphodiesterase of highest specific activity (Table 2) was that present in the peroxisomes (catalase activity 2.8 mmol/min/mg protein). The fraction containing intact chloroplasts and that containing Golgi bodies (inosine diphosphatase activity 2.5 μmol/min/mg protein) also possessed cyclic nucleotide phosphodiesterase activity with specific activity greater than unity. The microsomal fraction (NADH cytochrome *c* reductase activity 7.5 μmol/min/mg of protein) had the largest total activity of cyclic nucleotide phosphodiesterase. The major chlorophyll-containing fractions were located with bands of protein at densities of 1.18 and 1.12 g/ml, and had protein/chlorophyll ratios of 2.0 and 12.7, indicating an 'intactness' of ⩽ 10 and 86% [32], respectively. The 'intact' chloroplast fraction had a glyceraldehyde 3-phosphate dehydrogenase activity (stromal enzyme) of 4.83 nmol/min/mg of protein. This chloroplast fraction had a higher total phosphodiesterase activity and a 10-fold greater specific activity than that of the broken chloroplast fraction. The nuclear pellet had a DNA/RNA ratio of 3.0 and a DNA/protein ratio of 0.09. However, since its very low phosphodiesterase activity could be due to contamination of the nuclear

Table 2. Distribution of cyclic nucleotide phosphodiesterase in spinach leaf organelles

Fraction	Protein (mg/ml)	Specific activity (nmol/min/mg protein)	Total activity in fraction (nmol/min)
Crude homogenate	3.0	0.12	
10000 *g* Supernatant	1.5	0.22	6.9
Broken chloroplasts (density 1.18 g/ml)	0.50	0.27	4.2
Intact chloroplasts (density 1.121 g/ml)	0.20	3.30	20.7
Peroxisomes (density 1.26 g/ml)	0.05	11.0	17.3
Golgi bodies (density 1.17 g/ml)	0.10	4.69	14.7
Nuclei	0.65	0.04	0.9
Microsomes (density 1.17 g/ml)	1.02	0.98	31.4

Table 3. Substrate specificity of spinach cyclic nucleotide phosphodiesterases

Substrate (1 mM)	Phosphodiesterase 1_c (nmol/min/mg protein)	Phosphodiesterase 1_m (nmol/min/mg protein)
3′:5′-Cyclic AMP	0.80	0.50
3′:5′-Cyclic GMP	2.0	0.02
2′:3′-Cyclic AMP	0.02	1.35
2′:3′-Cyclic GMP	0.02	1.05

pellet with other subcellular particles, it was not investigated further.

Isoelectric focussing of the phosphodiesterase from the chloroplast fraction showed that the chloroplasts possess two bands of activity with isoelectric points of 3.7 and 6.3, respectively. These two bands were identified, by gel-filtration on Sephadex G-200 and anion-exchange chromatography on DEAE-cellulose, as peaks 2 and 1_c activities described above. Similar examination of the microsomal, peroxisomal and Golgi-containing fractions showed phosphodiesterases with isoelectric points at 3.7 and 4.7, identified, as before, as peaks 2 and 1_m, respectively.

Examination of the pH/activity profiles of preparations 1_m and 1_c showed that 1_m has an optimum at pH 4.9 whereas that of 1_c is at pH 6.1. Other differences between phosphodiesterases 1_c and 1_m were apparent from the effects of theophylline and of Ca^{2+} on these activities. Theophylline (0.1 mM) inhibited 1_c activity by 26% but 1_m by only 15%; the effects were reproducible. Caffeine had no effect on either. Ca^{2+} reproducibly stimulated 1_c activity by 32% but had no significant effect on 1_m activity. Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, NH_4^+, Fe^{3+} and Na^+, all at 0.1 mM, had no effect on either of the phosphodiesterase activities. As Table 3 shows, their substrate specificities are also divergent in that 1_c is more active towards 3′:5′-cyclic nucleotides than towards the 2′:3′-isomers and has a greater activity with guanine nucleotides, whereas 1_m is more active towards 2′:3′-cyclic nucleotides than towards the 3′:5′-isomers and has a greater activity towards adenine nucleotides.

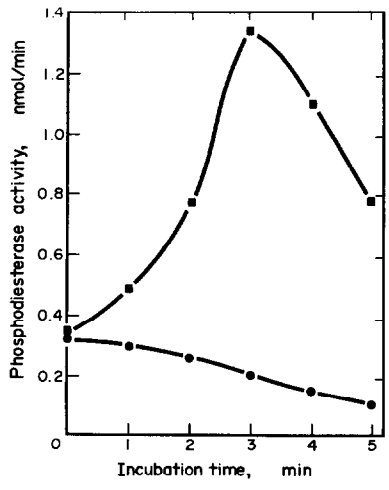

Fig. 1. Effect of trypsin upon cyclic nucleotide phosphodiesterase activity. Samples (1 mg) of peak 1_c protein (■) and peak 1_m protein (●) were separately incubated with trypsin (14 μg). The reactions were stopped by adding soybean trypsin inhibitor (28 μg). Details of the phosphodiesterase assay are given in the text.

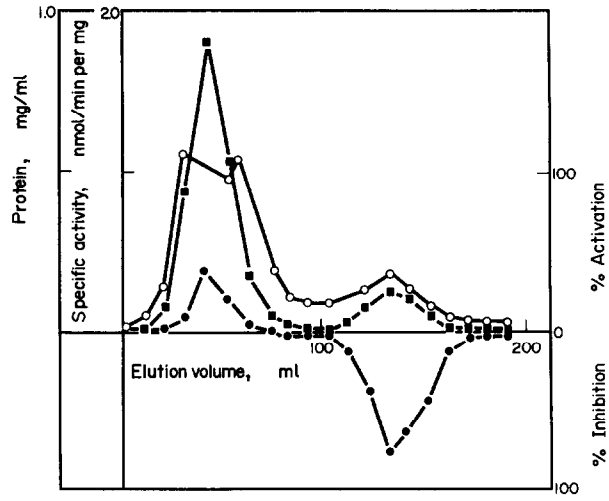

Fig. 2. Inhibition and activation of cyclic nucleotide phosphodiesterase activity by fractions from the chloroplasts of spinach leaves. Peak 1_c cyclic nucleotide phosphodiesterase, freed from effectors (see text), was assayed in the presence and absence of protein fractions obtained from spinach chloroplasts following gel-filtration chromatography on Sephadex G-100. In calculating % inhibition or activation, the intrinsic phosphodiesterase activity of the chloroplast fractions has been taken into consideration. Protein content (■), cyclic nucleotide phosphodiesterase activity (○), % inhibition or activation (●).

A further difference between peaks 1_c and 1_m was their response to incubation with trypsin (Fig. 1). Activity 1_m was inhibited, the extent of the inhibition being approximately proportional to the length of the incubation period. In contrast, during the initial stages of a 3 hr incubation of 1_c with trypsin, a 5-fold stimulation of activity was observed. Gel-filtration chromatography on Sephadex G-200 of 1_c that had been incubated with trypsin yielded a wide range of fractions possessing phosphodiesterase activity. One of these fractions was partially excluded from the gel, indicating a MW of at least 5×10^5. Another of the fractions had an apparent MW of 1.8×10^5. The main peak of phosphodiesterase activity eluted at a point indicating a MW of 3.7×10^4. These results showed that stimulation of peak 1_c by preincubation with trypsin was associated with a marked decrease in the MW of the cyclic nucleotide phosphodiesterase originally present.

Repeated freezing and thawing of preparations 1_c and 1_m also evoked a difference in response. Whereas peak 1_m was unaffected, the activity of peak 1_c towards cyclic AMP and cyclic GMP increased after the first cycle and continued up to a maximum of 5-times the original activity after three cycles. That the results of both the trypsin experiments and freeze-thawing were attributable to the removal of an inhibitor is indicated by the data in Fig. 2. These data show the effects upon phosphodiesterase of various protein fractions obtained by gel-filtration of a spinach extract. The inhibitor has an apparent MW of 8500 and under the experimental conditions, gave 70% inhibition of the partially purified cyclic nucleotide phosphodiesterase. A high MW fraction, possessing low enzymic activity, was obtained during separation of the inhibitor and observed to have a synergistic effect with the inhibitor-free phosphodiesterase (Fig. 2). The resulting overall increase in activity of 20% indicates the existence of an activator.

DISCUSSION

The production of both 3'- and 5'-AMP during hydrolysis of cyclic AMP by cyclic nucleotide phosphodiesterase from spinach is similar to that observed previously with other plant tissue [8, 9]. In contrast, cyclic nucleotide phosphodiesterases from animal sources hydrolyse cyclic AMP to yield 5'-AMP as the sole product. The unidentified radioactive product that was also produced from cyclic AMP-[8-^{3}H] during incubation with the crude plant enzyme may be related to the compound reported by Keates [14]. Association of the spinach phosphodiesterase with nucleotidase activity is as previously observed with other plant tissues [10].

The resolution by gel-filtration of the crude extracts of cyclic nucleotide phosphodiesterase from spinach into two peaks of activity was comparable to earlier findings with *Phaseolus* [13]. Although the peaks from *Phaseolus* were precipitated at concentrations of $(NH_4)_2SO_4$ which differed from those required with spinach extract, the MW of the peak 1 activity from both sources was comparable. However, the MW of peak 2 phosphodiesterase from spinach was only one-quarter of that of peak 2 phosphodiesterase from *Phaseolus*. The isoelectric focussing characteristics of peak 1_m and peak 2 are similar to those bands 1a and 1b from *Phaseolus* [13].

The observations with peaks 1_c and 1_m concerning isoelectric focussing behaviour, pH optima, substrate specificity, and sensitivity to metal ions and methylxanthines emphasize the differences between the two main phosphodiesterase activities. Peak 1_m, with its lower pH optimum, higher activity towards 2':3'-cyclic nucleotides, and lower sensitivity to theophylline, appears comparable to the enzyme from pea seedlings, described by Lin and Varner [8] as part of an RNA catabolizing mechanism rather than as part of a 3':5'-cyclic AMP orientated system. Since in the present work, 1_m was found in several extraplastidic sites, and as Lin and Varner used a gentler method of tissue disruption and included a 10000 *f*/10 min centrifugation step, it is possible that they discarded most of the peak 1_c activity in unbroken chloroplasts. Their phosphodiesterase preparation would therefore be expected to consist almost entirely of the 1_m type of activity.

Peak 1_c with its higher pH optimum, greater activity towards 3':5'-cyclic nucleotide substrates relative to their 2':3'-counterparts, and greater susceptibility to inhibition by theophylline, is far more comparable to the cyclic nucleotide phosphodiesterases isolated from bacteria and animal tissues. The presence of an endogenous associated inhibitor of phosphodiesterase is in agreement with the findings of Brewin and Northcote [11], and the presence of both an endogenous activator and inhibitor lends credibility to a cyclic AMP regulatory system in higher plants.

EXPERIMENTAL

Materials. Spinach seeds (*Spinacea oleracea* cv Viroflay) were soaked in running water for 48 hr and germinated in Levington potting compost. Seedlings were grown for 4 weeks at 20° in a cycle of 16 hr light (5 klx) and 8 hr dark. Upon harvesting, the plants were rinsed in H_2O, blotted dry and weighed. Plant material was kept at 4° for 30 min before homogenization.

Measurement of cyclic nucleotide phosphodiesterase activity. Two assay procedures were routinely used, a colorimetric method [15] and a radiochemical method. The latter was essentially that of ref. [16] with minor modifications. Extracts (100 μg protein/sample) were assayed in a reaction mixture containing 0.1 μCi cyclic AMP-[8-^{3}H] or the corresponding radioactive guanosine nucleotide, cyclic AMP or cyclic GMP (1 mM) and 40 mM Tris HCl buffer (pH 6.5) to a total vol. of 0.4 ml.

Liquid scintillation counting. The radioactivity of samples (0.5 ml) was measured by liquid scintillation counting using 5 ml scintillant containing 0.05% (w/v) 2,5-diphenyloxazole and 10% (w/v) naphthalene, both in dioxane. The scintillation spectrometer was set to a pre-set error of 0.2% and counting efficiency was 35%.

Estimation of chlorophyll and protein. Protein concentration was determined by the method of Lowry *et al.* [17]. For determination of chlorophyll, a sample of an appropriate organelle suspension (0.5 ml) was first shaken with 4.5 ml H_2O, 20 ml Me_2CO added and the shaking continued. After filtering though sintered glass (por. 1) the A_{649} and A_{665} of the filtrate were measured and the chlorophyll content calculated from these values [18].

Estimation of organelle marker enzymes. NADH-cytochrome *c* reductase activity was measured spectrophotometrically from the increase in A_{550} [19]. Cytochrome *c* oxidase was determined by the method of ref. [20]. Excess sodium dithionite was oxidized as recommended by ref. [21]. In order to measure catalase activity, samples (0.2 ml) of organelle suspension were added to 2.8 ml 0.15 M Na-Pi buffer (pH 7) containing 12.5 mM H_2O_2 and the rate of decrease in A_{240} was monitored. Acid phosphatase in organelle suspensions (0.1 ml samples) was assayed using as substrate 5.5 mM *p*-nitrophenylphosphate in 0.05 M sodium citrate buffer (pH 4.8); the final incubation vol. was 1 ml. The reaction mixture was equilibrated at 37° for 10 min before addition of the enzyme sample. After incubation at 37° for a further 30 min, the reaction was terminated by adding 0.4 ml 0.1 M NaOH and the A_{400} measured against a reagent blank. Determination of fructose 1,6-diphosphatase activity was by the method of ref. [22]. The Pi liberated was measured by the procedure of ref. [23]. For estimation of $NADP^+$ glyceraldehyde phosphate dehydrogenase, an organelle sample containing 0.2 mg protein was added to a reaction mixture comprising 20 μmol $MgSO_4$, 13 μmol cysteine, 2.6 μmol glutathione, 22.5 μmol phosphoglyceric acid, 2.75 μmol ATP and 0.5 M Tris–HCl buffer (pH 7.5) to a final vol. of 0.2 ml. The reaction was started by adding 0.4 μmol NADP and the A_{340} measured at 30 sec intervals. Phosphoglyceric acid was omitted from the controls. In some assays, excess phosphoglycerate kinase was added as it was not present in the crude protein extract. For glucose 6-phosphatase, the activity of 10 μl samples was measured by the method of ref. [22].

Extraction and estimation of nucleic acids. Nucleic acids were extracted in 0.5 M $HClO_4$ at 70° [24, 25]. DNA was determined by the diphenylamine method [26, 27] and RNA by the orcinol procedure [28].

Ultrafiltration. Protein fractions were concd by ultrafiltration using Amicon UM 10 membranes in conjunction with a model 52 stirred cell (65 ml capacity). Pressure was supplied by N_2 at 70 MPa. Where necessary, further concn was effected using a Model 12 stirred cell (10 ml capacity).

Zonal centrifugation. For zonal centrifugation, a rotor type JCF-Z was used in conjunction with a model J-21 prep. centrifuge (Beckman Ltd.). Fractions (10 ml) were automatically collected from the rotor at 2500 *g*; protein was detected by monitoring at 279 nm. The density of sucrose gradients was monitored at 5° with a refractometer.

Isoelectric focussing. Isoelectric focussing was as previously described [13]. At the end of protein separations by this

method, the pH gradient was determined using a surface pH electrode. The gel was cut into 5 mm squares and each square was eluted with 1 ml 40 mM Tris–HCl buffer (pH 6.5) for 12 hr at 4°. Each eluate was assayed for cyclic nucleotide phosphodiesterase activity. Other gel slices were stained for protein with Coomassie brilliant blue [19].

Preparation of cell-free extract. Using an ice-cold mortar and pestle, spinach leaf tissue (300 g) was macerated in 300 ml 40 mM Tris–HCl buffer (pH 7.4) with acid-washed sand. The preparation was filtered through muslin to remove coarse debris and the filtrate (345 ml) sonicated (20 kHz/2.5 min). After centrifuging at 100000 *g* for 90 min, the supernatant was dialysed against 20 vol. of the same buffer for 12 hr. The non-diffusible fraction was centrifuged at 10000 *g* for 10 min to remove the ppt. formed during dialysis and the supernatant examined for cyclic nucleotide phosphodiesterase activity.

Examination of products of the enzymic hydrolysis of cyclic AMP. The hydrolysis products of 3′:5′-cyclic AMP by cyclic nucleotide phosphodiesterase from spinach were separated and identified by TLC on activated Si gel G using the solvent system *iso*-PrOH–NH_3 soln (s.g. 0.88)–H_2O (7:1:2). Samples (10 µl) were run for 4 hr at room temp., plates were dried in air and spots visualized in UV light prior to examination with a 'Panax' radiochromatogram scanner. Adenosine, 3′-AMP, 5′-AMP and 3′:5′-cyclic AMP were used as internal standards. In this study of the hydrolysis products, the reaction mixture contained 2 µmol of 3′:5′-cyclic AMP-[8-^{3}H] in 40 mM Tris–HCl buffer (pH 7.4) and the protein fraction (0.5 mg) in a total vol. of 1 ml. A control incubation was also set up from which the enzyme had been omitted. After incubation at 37° for 12 hr, the reaction mixture was divided into two portions, one was incubated with a mixture of 3′- and 5′-nucleotidase (100 µg of each) and the other with 40 mM Tris–HCl buffer at pH 7.4 (0.1 ml). Both portions were incubated at 37° for 60 min.

Purification of cyclic nucleotide phosphodiesterase. The 100000 *g* supernatant from the spinach leaf homogenate was fractionated by precipitation with $(NH_4)_2SO_4$ and the protein fraction obtained at 20–40% saturation dissolved in *ca* 10 ml 40 mM Tris–HCl buffer (pH 7.4) and dialysed for 24 hr against 20 vol. of the same buffer. During dialysis, the buffer was changed 3 times. The dialysed protein fraction was centrifuged at 10000 *g* for 10 min to remove ppt. and a sample of the supernatant containing 500 mg protein was taken for gel-filtration on a column (45 × 2.5 cm dia) of Sephadex G-200. The column was eluted with 40 mM Tris–HCl buffer (pH 7.4) at a flow rate of 25 ml/hr. Fractions (10 ml) were collected and examined for phosphodiesterase activity and protein content. The apparent MWs of the proteins were determined by the method of ref. [30]. The fractions comprising the first protein peak (peak 1) from the gel-filtration column were pooled and a sample containing 20 mg of protein was concd by ultrafiltration. The concentrate was introduced onto a column (10 × 0.5 cm dia) of DEAE-cellulose and eluted with 50 ml 40 mM Tris–HCl buffer (pH 7.4) followed by a stepwise gradient of 0.125, 0.250, 0.375 and 0.5 M NaCl in the same buffer. Fractions (1 ml) were collected and their protein contents and cyclic nucleotide phosphodiesterase activities determined. The phosphodiesterase activities 1_c, 1_m, and 2 obtained from the DEAE-cellulose column (Table 1) were further purified by isoelectric focussing in a linear pH gradient of pH 3.5–10.0.

Subcellular fractionation. Peroxisomes and microsomes. The tissue homogenate was prepared by cutting a 100 g sample of destarched plants into small pieces with scissors, then disrupting by gentle stirring with a plastic spatula in ice-cold 40 mM Tris–HCl buffer (pH 7.4) containing 0.8 M sucrose. Centrifugation was used to remove coarse debris (600 g/10 min) and chloroplasts (1000 g/10 min). The supernatant (250 ml) was layered over a gradient composed of 1.3 M sucrose (200 ml), 1.5 M sucrose (400 ml), 1.8 M sucrose (400 ml) and 2.3 M sucrose (250 ml). The gradient was supported by 2.4 M sucrose (150 ml). The sample was moved away from the rotor cone with a 10% (w/v) sucrose overlay (250 ml). After centrifuging at 40000 *g* for 6 hr at 4°, the rotor was decelerated to 3000 *g* and the developed gradient was removed from the centre of the rotor by pumping in 2.4 M sucrose at the periphery. Fractions (10 ml) were collected and monitored for protein, chlorophyll, cyclic nucleotide phosphodiesterase and marker enzymes. The peroxisomal and microsomal fractions obtained were further purified by separately centrifuging them in a linear density gradient of sucrose (1.15–1.35 g/ml) with a 2.4 M sucrose cushion. The fractions (8.5 ml of each) were layered over the gradient and centrifuged at 105000 *g* for 3 hr. The resulting fractionation was again monitored for protein, chlorophyll, phosphodiesterase and marker enzymes. Fractions containing peroxisomes were identified by their catalase activity and those containing microsomes by their activity of Antimycin-insensitive NADH-cytochrome *c* reductase.

Mitochondria. The homogenate, prepared as for isolation of peroxisomes, was centrifuged at 250 *g* for 90 sec to remove nuclei and coarse debris. After removing chloroplasts at 1000 *g*/5 min, a 10000 *g*/10 min fraction was collected. This contained mitochondria, broken chloroplasts and peroxisomes. The pellet was resuspended in homogenization medium (20 ml) and a sample (8.5 ml) layered over a linear density gradient of sucrose (1.15–1.35 g/ml) for centrifuging at 105000 *g*. Fractions (1 ml) were collected and assayed for marker enzymes, chlorophyll, protein, and cyclic nucleotide phosphodiesterase activity. The mitochondrial fraction was characterized by cytochrome oxidase activity and Antimycin-sensitive NADH-cytochrome *c* reductase.

Golgi fraction. The spinach homogenate was centrifuged at 400 *g*/30 min and the supernatant (32.5 ml) layered over a discontinuous density gradient of 1.6 M sucrose (5 ml), and 1.8 M sucrose (2.5 ml). After centrifuging at 12000 *g* for 30 min, the fraction at the 1.6 M sucrose interface (10 ml) was collected, layered onto a gradient consisting of 1.8 M sucrose (2.5 ml), 1.6 M (5.0 ml), 1.5 M (10.8 ml) and 1.25 M (10 ml), and centrifuged at 105000 *g* for 3 hr. Fractions (0.5 ml) were assayed for protein, chlorophyll, marker enzymes and phosphodiesterase activity. Fractions containing Golgi bodies were identified by latent inosine diphosphatase activity.

Nuclei. Nuclei were isolated by the method of ref. [31].

Chloroplasts. Intact chloroplasts were obtained by a modification of the rate-zonal centrifugation method of Leech [32]. After removing coarse debris and nuclei by centrifuging at 600 *g*/2 min, the spinach homogenate (26 ml) was layered onto a discontinuous density gradient consisting of 41% (w/v) sucrose (6 ml) on top of 50% (w/v) sucrose (10 ml), all in 40 mM Tris–HCl buffer (pH 7.4). After centrifuging at 1000 *g*/20 min in a Beckman JS-13 swing-out rotor, fractions were collected and assayed for protein, chlorophyll, marker enzymes and cyclic nucleotide phosphodiesterase. Fractions containing intact chloroplasts were identified by their glyceraldehyde 3-phosphate dehydrogenase activity; 'intactness' was assessed from the protein/chlorophyll ratio [33].

pH Optima. The buffers used to cover the pH range required were 50 mM HOAc–NaOH (pH 3.0–5.8), 50 mM cacodylate–HCl (pH 5.0–7.2) and 50 mM Tris–HCl (pH 7.0–9.0).

Freezing and thawing of enzyme preparations. The peaks 1_c and 1_m cyclic nucleotide phosphodiesterase activities were frozen at −20° for 12 hr. After thawing, a ppt. that had formed was removed by centrifuging. This freeze–thaw process was re-

peated ×6 and the enzymic activity measured at each stage using both cyclic AMP and cyclic GMP as substrates.

Separation of cyclic nucleotide phosphodiesterase inhibitor and activator from chloroplasts. A suspension of intact chloroplasts (see above) was sonicated at 20 kHz for 3 min, then centrifuged; the supernatant was dialysed for 12 hr at 4° against 40 mM Tris–HCl buffer at pH 7.4. After dialysis, the non-diffusible fraction was adjusted to pH 5.0 with cacodylate–HCl buffer (0.1 M; pH 5.0) to remove the inhibitor from the cyclic nucleotide phosphodiesterase. The ppt. that formed was removed by centrifuging at 10000 *g* for 20 min. The supernatant was fractionated on a column (45 × 2.5 cm dia) of Sephadex G-100 using 40 mM cacodylate–HCl buffer (pH 5.8) as the eluting buffer. Fractions (5 ml) were collected and assayed for protein and cyclic nucleotide phosphodiesterase activity. The inhibitory and stimulatory fractions were detected by examining 50 µl samples for effect on the phosphodiesterase activity of 50 µl portions of peak 1_e that had been frozen and thawed ×5 in order to destroy the inhibitor.

Trypsin treatment of enzyme activity. Peaks 1_c and 1_m (1 mg samples) were separately incubated with trypsin (14 µg) at 25° for 5 min. Soybean trypsin-inhibitor (28 µg) was added to stop the reactions and the incubates were assayed for phosphodiesterase activity.

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