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THE CYCLIC NUCLEOTIDE PHOSPHODIESTERASES OF SPINACH CHLOROPLASTS AND MICROSOMES

ERIC G. BROWN, MALCOLM J. EDWARDS, RUSSELL P. NEWTON and CHRISTOPHER J. SMITH

Department of Biochemistry, University College of Swansea, Swansea SA2 8PP, Wales, U.K.

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Key Word Index—*Spinacea oleracea*; Chenopodiaceae; spinach; cyclic nucleotides; phosphodiesterase; chloroplasts; microsomes.

Abstract—Cyclic nucleotide phosphodiesterase was extracted from intact chloroplasts and partially purified. Peak 1_c activity from Sephadex G-200 was resolved by electrophoresis into two major bands (MWs 1.87×10^5 and 3.7×10^5). Both also possessed acid phosphatase, ribonuclease, nucleotidase and ATPase. The chloroplast peak 1_c cyclic nucleotide phosphodiesterase was located in the envelope. Peak 1_m cyclic nucleotide phosphodiesterase obtained from the microsomal fraction had a MW of 2.63×10^5. Electrophoresis separated 1_m into two bands of cyclic nucleotide phosphodiesterase activity (MWs 2.63×10^5 and 1.28×10^5). Both contain ATPase, ribonuclease, nucleotidase, but not acid phosphatase. Peak 1_c has high activity towards 3′:5′-cyclic AMP and 3′:5′-cyclic GMP but little towards 2′:3′-cyclic nucleotides. Peak 1_m showed most activity towards 2′:3′-cyclic AMP, 2′:3′-cyclic GMP and 2′:3′-cyclic CMP with little activity towards 3′:5′-cyclic nucleotides. With 1_c, 3′:5′-cyclic AMP and 3′:5′-cyclic GMP exhibit mixed-type inhibition towards one another. The 2′:3′-cyclic AMP phosphodiesterase 1_m was competitively inhibited by 2′:3′-cyclic GMP. *p*-Chloromercuribenzoate inhibits 1_c but not 1_m. Electrophoresis after dissociation indicates that 1_c and 1_m are both enzyme complexes. After dissociation, the 1_c complex but not that of 1_m could be reassociated. The ribonuclease of the 1_m complex hydrolyses RNA to yield 2′:3′-cyclic nucleotides as the main products. These results are compatible with the 1_c cyclic nucleotide phosphodiesterase complex being involved in the metabolism of 3′:5′-cyclic AMP, and the 1_m complex being concerned with RNA catabolism.

INTRODUCTION

The presence of 3′:5′-cyclic AMP in the tissues of higher plants was suggested by the work of Pollard [1] and confirmed by Brown and Newton [2] and Raymond *et al.* [3]; nevertheless some authors [4, 5] have argued against these findings. However, recent work involving the specific binding-protein procedure [6] and the protein kinase method [7] has further substantiated the presence of this nucleotide in higher plants. Furthermore, a protein which specifically binds cyclic AMP has been detected in plant tissues [8]. One of the arguments that has been used [9, 10] against a possible physiological role for cyclic AMP in higher plants is that a plant phosphodiesterase which can hydrolyse this nucleotide *in vitro* has markedly different properties to the cyclic AMP phosphodiesterase of animals and micro-organisms. In particular, as the plant enzyme studied by these authors was more effective with 2′:3′-cyclic AMP as substrate, they suggested that its physiological role is in RNA catabolism rather than in a 3′:5′-cyclic AMP regulated system. Recent studies in this laboratory [11] have, however, demonstrated the plurality of cyclic nucleotide phosphodiesterase activity in plant tissues. One of these activities is specifically located in the chloroplasts and exhibits greater activity towards 3′:5′-cyclic AMP than towards 2′:3′-cyclic AMP. Another, located in peroxisomes, microsomes and Golgi bodies, shows a converse specificity. With the aim of clarifying these arguments concerning the cyclic nucleotide phosphodiesterase activity of higher plants, we sought to purify the enzymes concerned and examine their properties.

RESULTS

An intact chloroplast fraction was obtained from spinach leaves by sucrose density gradient centrifugation as described in the Experimental. The identity of the fraction was confirmed by its density (1.21 g/ml) and by its glyceraldehyde 3-phosphate dehydrogenase activity (483 nmol/min/mg protein) together with a protein/chlorophyll ratio of 12.7. The latter indicates 86% of intact chloroplasts [12]. Purification of the cyclic nucleotide phosphodiesterase from this chloroplast fraction is outlined in Table 1. Chloroplasts isolated by the procedure described had a cyclic nucleotide phosphodiesterase acitivity at least 10-times greater than that of chloroplasts isolated by a variety of other published methods.

Following fractional precipitation with $(NH_4)_2SO_4$, 97% of the total recovered cyclic nucleotide phosphodiesterase activity was in the 20–40% fraction. This fraction was chromatographed on a column of

Table 1. Purification of cyclic nucleotide phosphodiesterase from spinach chloroplasts

	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Relative purity	Recovery %
Chloroplast					
105 000 g supernatant	150	90	0.60	1.0	—
20–40% satd. $(NH_4)_2SO_4$ ppt.	68.0	272.4	4.0	6.7	100
Sephadex G-200	12.0	264.0	22.0	36.6	96.9
DEAE-cellulose	1.20	43.2	36.0	60.0	15.8
Electrophoresis 5% polyacrylamide gel					
Band 1	0.02	5.91	295.5	492.5	2.17
Band 2	0.02	4.35	217.5	362.5	1.60

Sephadex G-200 as previously described [11] and the activity constituting peak 1_c collected. It had an apparent MW of 5.0×10^5 and in addition to cyclic nucleotide phosphodiesterase activity, also possessed acid phosphatase, ATPase, ribonuclease and nucleotidase activities. When fraction 1_c was subjected to chromatography on a column of DEAE-cellulose, 96% of the cyclic nucleotide phosphodiesterase activity eluted in the buffer wash and was again accompanied by acid phosphatase, ATPase, ribonuclease, and nucleotidase activities. Resolution of this fraction by polyacrylamide gel electrophoresis produced two major bands of cyclic nucleotide phosphodiesterase activity (MWs 1.87×10^5 and 3.7×10^5). Both bands exhibited acid phosphatase, ribonuclease, nucleotidase and ATPase activities.

Subfractionation of the disrupted chloroplasts in a sucrose density gradient yielded three discrete fractions (Table 2) containing, respectively, stroma, thylakoid membranes, and envelope membranes. Only the latter fraction could be demonstrated to possess cyclic nucleotide phosphodiesterase activity.

Purification of the cyclic nucleotide phosphodiesterase activity from the microsomal fraction of spinach leaves is shown in Table 3. During the initial fractional precipitation with $(NH_4)_2SO_4$, 89% of the total recovered activity was in the 20–40% saturation fraction. Resolution of this by gel-filtration on Sephadex G-200, using the procedure described previously [11], yielded an activity (peak 1_m) with a MW of 2.63×10^5. During chromatography of this on a column of DEAE-cellulose, the cyclic nucleotide phosphodiesterase eluted with 0.375 M NaCl and was accompanied by acid phosphatase, ATPase, ribonuclease, and nucleotidase activities. Further fractionation by polyacrylamide gel electrophoresis resulted in two bands of cyclic nucleotide phosphodiesterase activity. One of these had a MW of 2.63×10^5, and the other of 1.28×10^5. As with the bands from the chloroplasts, both contained ATPase, ribonuclease, and nucleotidase but unlike the chloroplast bands, no acid phosphatase could be detected.

The microsomal fraction was solubilized with 0.1% (w/v) sodium deoxycholate and centrifuged. Both pellet and supernatant were examined for protein, RNA and cyclic nucleotide phosphodiesterase contents. The results indicated that the cyclic nucleotide phosphodiesterase activity is located in the microsomal membranes (Table 4).

Table 5 shows the polar lipid composition of the chloroplast envelope fraction, the microsome fraction, and peak 1_c isolated by gel-filtration on Sephadex. Although there are differences between the amounts of individual polar lipids in the chloroplast envelope and those in the peak 1_c preparations, the major polar lipids in both are galactolipids. In contrast, the major polar lipid components of the microsomal fraction are phosphatides.

The substrate specificity of the cyclic nucleotide phosphodiesterase from peak 1_c and that from peak 1_m is shown in Table 6. The enzyme from peak 1_c had high activity towards 3′:5′-cyclic GMP and 3′:5′-cyclic

Table 2. Subfractionation of spinach chloroplasts in a sucrose density gradient and identification of fractions by their marker enzymes

Fraction	Position in sucrose gradient	Cyclic nucleotide phosphodiesterase activity (nmol/min/mg)	Fructose 1,6-diphosphatase activity (μmol/min/mg)	Trypsin-activated Ca^{2+}-dependent ATPase (μmol/min/mg)	Mg^{2+}-dependent ATPase (μmol/min/mg)
Stroma	0.6 M sucrose interface	0	10.3	0	0.2
Thylakoid membranes	1.2 M sucrose interface	0	0.8	47.0	0
Envelope	0.93 M sucrose interface	14.2	0	0	14.6

Table 3. Purification of cyclic nucleotide phosphodiesterase from spinach microsomes

Step	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Relative purity	Recovery %
Microsome 100 000 g extract	150	42.4	0.28	1.0	—
20–40% satd. $(NH_4)_2SO_4$ ppt.	70.2	209.6	3.0	10.7	100
Sephadex G-200	12.3	169.6	13.8	49.3	80.9
DEAE-cellulose	1.1	29.3	26.6	95.0	14.0
Electrophoresis 5% polyacrylamide gel					
Band 1	0.02	6.7	335.0	1196.4	3.2
Band 2	0.02	4.14	207.0	739.9	2.0

Table 4. Distribution of cyclic nucleotide phosphodiesterase activity after solubilization of microsomes with 0.1% (w/v) sodium deoxycholate

Fraction	Protein (mg)	RNA (mg)	RNA: protein	Cyclic nucleotide phosphodiesterase (nmol/min/mg)
Pellet 20 000 g	3.0	9.6	3.2	0.02
Supernatant 20 000 g	18.2	0.5	0.02	6.60

AMP but little activity towards the corresponding 2′:3′-cyclic nucleotides. Peak 1_c also hydrolysed 3′:5′-cyclic TMP and 3′:5′-cyclic dTMP but had no activity with either the 2′:3′-, or the 3′:5′-cyclic nucleotides of cytosine, uracil and xanthine. Additionally, peak 1_c exhibited ATPase, phosphatase and ribonuclease activities. The cyclic nucleotide phosphodiesterase from peak 1_m showed most activity towards 2′:3′-cyclic AMP, 2′:3′-cyclic GMP and 2′;3′-cyclic CMP, with relatively little activity towards the 3′:5′-cyclic nucleotides. The peak 1_m fraction also exhibited some activity towards ATP, 3′-AMP and yeast RNA but did not hydrolyse *p*-nitrophenyl phosphate.

A Lineweaver–Burk plot of kinetic data for peak 1_c with 3′:5′-cyclic AMP and 3′:5′-cyclic GMP as substrate and inhibitor, respectively, showed an intersection at an extrapolation point with a positive ordinate value and a negative abscissa value, indicating a mixed-type inhibition. Similar plots of kinetic data for peak 1_m with 2′:3′-cyclic AMP and 2′:3′-cyclic GMP

Table 5. Comparison of the polar lipid composition of the peak 1_c complex with those of the chloroplast and microsome preparations

	mg/100 mg of total polar lipid		
Polar lipid component	Peak 1 complex	Chloroplast envelope preparation	Microsomal preparation
Monogalactosyl diglyceride	23.4	25.7	7.6
Digalactosyl diglyceride	32.5	39.8	0.0
Sulpholipid	9.7	16.8	0.0
Phosphatidylcholine	23.4	15.3	42.9
Phosphatidylethanolamine	0.0	0.0	34.2
Phosphatidylglycerol	11.0	2.4	2.0
Phosphatidylserine	0.0	0.0	9.8
Phosphatidylinositol	0.0	0.0	3.5

The peak 1_c complex was obtained from the Sephadex G-200 gel-filtration stage of the partial purification outlined in Table 1.

Table 6. Substrate specificity of spinach cyclic nucleotide phosphodiesterases

Cyclic nucleotide substrate	Peak 1_c phosphodiesterase (nmol/min/mg)	Peak 1_m phosphodiesterase (nmol/min/mg)
3′:5′-cAMP	0.80	0.50
2′:3′-cAMP	0.02	1.35
3′:5′-Cyclic dAMP	0.20	0.03
3′:5′-cCMP	0.00	0.02
2′:3′-cCMP	0.00	0.65
3′:5′-Dibutyryl cAMP	0.00	0.03
3′:5′-cGMP	2.0	0.02
2′:3′-cGMP	0.02	1.05
3′:5′-cIMP	0.00	0.01
2′:3′-cIMP	0.00	0.00
3′:5′-cTMP	0.30	0.00
3′:5′-Cyclic dTMP	0.20	0.04
3′:5′-cUMP	0.00	0.02
3′:5′-cXMP	0.00	0.03

All the substrates were examined at a final concentration of 1 mM.

as substrate and inhibitor, respectively, showed an intersection on the ordinate axis, indicating competitive inhibition. A further difference between the 1_c and 1_m cyclic nucleotide phosphodiesterase activities was seen in their respective sensitivities to *p*-chloromercuribenzoate (Table 7). *p*-Chloromercuribenzoate inhibited peak 1_c activity but this activity was restored by 2-mercaptoethanol. Peak 1_m showed negligible sensitivity to *p*-chloromercuribenzoate.

Fractionation of peak 1_m by polyacrylamide gel electrophoresis produced two major bands, each of which contained cyclic nucleotide phosphodiesterase, ATPase and ribonuclease activity. After exposing peak 1_m activity to five cycles of freezing and thawing followed by electrophoresis, these two protein bands were partially dissociated and three new bands of protein of lower MWs appeared. The three new bands contained, in order of increasing mobility towards the cathode, ATPase, cyclic nucleotide phosphodiesterase and ribonuclease.

Polyacrylamide gel electrophoresis of peak 1_c on 5% gels produced two high MW protein bands, both of which contained cyclic nucleotide phosphodiesterase, ATPase, ribonuclease, and acid phosphatase activities. After treatment of 1_c with 4 M urea for 60 min at 25°, the two high MW protein bands were replaced by four protein bands of lower MW. In order of decreasing MW, these bands contained ATPase, acid phosphatase, cyclic nucleotide phosphodiesterase and ribonuclease activity. The nucleotidase activity was located in the ATPase protein band. After acid-dissociation of 1_c, reassociation was effected by incubating a neutralized sample at pH 7.4 for 60 min. Fractionation of this reassociated sample, by electrophoresis in 5% polyacrylamide gel, produced one high MW protein band and three of lower MW. The high MW band corresponds to the lighter of the two original high MW bands, obtained from undissociated 1_c, and containing cyclic nucleotide phosphodiesterase, ATPase, ribonuclease and acid phosphatase activity. Of the other three bands, one contained ATPase, another contained acid phosphatase and cyclic nucleotide phosphodiesterase, and the third contained cyclic nucleotide phosphodiesterase and ribonuclease.

Examination of the hydrolytic activity of peaks 1_c and 1_m towards yeast RNA revealed a further difference between these two fractions. Peak 1_m ribonuclease activity produced 3′-GMP as the sole product

Table 7. Effect of *p*-chloromercuribenzoate followed by 2-mercaptoethanol on the 1_c and 1_m cyclic nucleotide phosphodiesterases

Treatment	Cyclic nucleotide phosphodiesterase activity (nmol/min/mg)	
	Peak 1_c	Peak 1_m
1. None (control)	40.0	21.4
2. Pre-incubation with *p*-chloromercuribenzoate (1 mM) for 3 min at 37°	17.4	20.6
3. Treatment 2 followed by 10 min at 37° with 2-mercaptoethanol (10 mM)	37.8	21.2

Table 8. Selectivity of the ribonuclease activities of the peak 1_c and peak 1_m complexes

Hydrolysis by:	Nucleotides released from RNA (mol %)			
	A	U	G	C
KOH (control)	34.0	30.2	23.5	12.3
Peak 1_c complex	33.8	0.0	0.0	66.2
Peak 1_m complex	23.3	17.8	58.9	0.0

Conditions used for the chemical and enzymic hydrolyses are detailed in the text.

after 3 hr, but after incubation for 6 hr 3′-AMP was released together with small amounts of 3′-UMP. Absence of a lag phase and the nature of the products suggest that this enzyme is an endonuclease [13]. Hydrolysis of yeast RNA by peak 1_c ribonuclease activity yielded 3′-CMP after 3 hr, with small amounts of 3′-AMP appearing after 6 hr. This indicates that 1_c has a purine specificity: the nature of the products points to it being an endonuclease [13]. Comparison of the bases released from yeast RNA by the ribonucleases of peaks 1_c and 1_m with the base frequency of the RNA indicate the selective release of specific nucleotides by these enzymes (Table 8).

Hydrolysis of yeast RNA by 1_m ribonuclease, freed from contaminating enzymes after dissociation of the complex, yielded as the major products 2′:3′-cyclic GMP (68.8 mol %), 2′:3′-cyclic AMP (25.3 mol %) and 2′:3′cyclic UMP (5.9 mol %). No 2′:3′-cyclic CMP was detected. The effect of various end products is shown in Table 9; 3′-AMP, 3′-GMP, 2′:3′-cyclic AMP, 3′:5′-cyclic AMP, 2′:3′-cyclic GMP, 3′:5′-cyclic GMP, ADP and ATP all inhibit the ribonuclease activity. None of the nucleotides, including cyclic nucleotides, of uracil and cytosine, had an inhibitory effect.

DISCUSSION

The relatively high level of cyclic nucleotide phosphodiesterase activity (1_c) found in intact chloroplasts in contrast to that in broken plastids, indicates that the enzyme is located either in the stroma or in the chloroplast envelop since both of these are absent in broken chloroplast preparations. Subfractionation of the chloroplasts and isolation of the chloroplast envelopes showed that the enzyme is present at this site. The peak 1_m activity is also membrane bound, being located in the microsomal membranes. Although the polar lipid composition of the peak 1_c complex indicates a relationship to the chloroplast envelope rather than to the microsomal membranes, this does not preclude the peaks 1_c and 1_m phosphodiesterases having the same function. However, the indications of our earlier work, which showed differing pH optima, sensitivity to metal ions and to trypsin [11], are confirmed in that there is a large difference in specificity of 1_m and 1_c, with 1_c showing greater activity towards 3′:5′-cyclic nucleotides than towards 2′:3′-cyclic nucleotides, whereas the converse is true of 1_m. Kinetic analysis of these activities shows that, with 1_c, 3′:5′-cyclic AMP and 3′:5′-cyclic GMP exhibit mixed-type

Table 9. Inhibition of peak 1_m ribonuclease by end products and related nucleotides

End product or related nucleotide	Inhibition of ribonuclease (% inhibition of control)
None (control)	0
3′-AMP	56.7
3′-GMP	43.5
3′-CMP	0.0
3′-UMP	0.0
2′:3′-Cyclic AMP	46.0
2′:3′-Cyclic GMP	35.4
2′:3′-Cyclic CMP	0.0
3′:5′-Cyclic AMP	52.3
3′:5′-Cyclic GMP	46.7
3′:5′-Cyclic CMP	0.0
ADP	74.2
ATP	89.8

All the compounds were examined at a final concentration of 1 mM.

inhibition towards one another. This suggests that both of these 3′:5′-cyclic nucleotides are substrates but that they bind, independently of each other, to different sites on the enzyme. This causes inhibition when both are present together in the reaction mixture. The 2′:3′-cyclic AMP phosphodiesterase activity of 1_m was shown to be competitively inhibited by 2′:3′-cyclic GMP, i.e. the latter nucleotide prevents 2′:3′-cyclic AMP from binding to the same site.

The marked difference in properties between 1_c and 1_m are further emphasized by their respective responses to *p*-chloromercuribenzoate. The reversible inhibition produced by *p*-chloromercuribenzoate and its alleviation by 2-mercaptoethanol also indicates that the 1_c cyclic nucleotide phosphodiesterase activity has sulphydryl group(s) involved in the catalytic process.

Electrophoresis after dissociation showed that both 1_m and 1_c cyclic nucleotide phosphodiesterases are associated with other enzyme activities, as has been suggested for other plant phosphodiesterases [14, 15]. However, acid phosphatase (present in 1_c only), nucleotidase, ribonuclease and ATPase are separable from cyclic nucleotide phosphodiesterase activity, indicating a multiplicity of enzymes associating together rather than a non-specific hydrolase. Although the reassociation of the chloroplast enzyme aggregate indicates that the aggregate is a functional multienzyme complex, the ease with which the dissociated system can be reconstituted demonstrates that, even if the enzymes exist free and separate *in vivo*, they can form an aggregate during the isolation procedure. That the complex exists as such *in vivo* is, however, indicated by the observation that during the isolation procedure the free enzyme could not be readily demonstrated. Furthermore, the enzymes concerned are impossible to purify by techniques other than those which neutralize non-covalent forces, so supporting the hypothesis that this enzyme aggregate is a functional multienzyme complex.

The peak 1_m cyclic nucleotide phosphodiesterase, although apparently not capable of reassociation, appears to be an aggregate of similar type. The ribonuclease present in this fraction hydrolysed yeast RNA liberating 2′:3′-cyclic AMP and 2′:3′-cyclic GMP as the major products; the cyclic nucleotide phosphodiesterase had greater activity with these two cyclic nucleotides than with their 3′:5′-counterparts. The nucleotidase also present in the aggregate is capable of further degrading these to adenosine and guanosine. Thus the properties of 1_m cyclic nucleotide phosphodiesterase are compatible with a role in the degradation of RNA, as suggested by Lin and Varner [9] for the phosphodiesterase they obtained from pea seedlings. In addition, since 3′-AMP, 3′-GMP, the 2′:3′- and 3′:5′-cyclic nucleotides of guanosine and adenosine, and ATP are all inhibitors of 1_m ribonuclease, this complex could provide a means of regulating *m*RNA degradation. Indeed, high levels of cyclic AMP would serve as a regulator of this process.

The relatively low activity of the 1_c complex with 2′:3′-cyclic nucleotides is indicative that the role of its phosphodiesterase is not related to RNA metabolism. On the contrary, the much greater activity with 3′:5′; cyclic nucleotides points to an involvement in the metabolism of 3′:5′-cyclic AMP and possibly cyclic GMP, since the presence of the latter in *Nicotiana tabacum* has been reported [16]. The existence of nucleotidase activity [14] and acid phosphatase [15] in a complex with cyclic nucleotide phosphodiesterase has been reported but the function of the complex remains undetermined.

EXPERIMENTAL

Materials. Spinach seeds (*Spinacea oleracea* cv Viroflay) supplied by Hurst, Gunson and Taber Ltd., Witham, Essex, were germinated, and the seedlings grown as previously described [11].

Measurement of cyclic nucleotide phosphodiesterase activity. Two procedures were routinely employed, both utilized measurement of reaction products. The colorimetric method has been previously described [17]. The radioactive method was essentially the method of Thomson and Appelman [18] with minor modifications. Enzyme samples containing 100 μg of protein were assayed in a reaction mixture containing 0.1 μCi of 3′:5′-cyclic AMP-[8-^{3}H] or 3′:5′-cyclic GMP-[8-^{3}H], 3′:5′-cyclic AMP (1 mM) or 3′:5′-cyclic GMP (1 mM) and 40 mM Tris–HCl buffer (pH 6.5), all in a total vol. of 0.4 ml. Radioactivity was measured by liquid scintillation counting as described previously [17].

Determination of protein and chlorophyll concentrations. The method of Lowry *et al.* [19] was used for determining protein concentration. The chlorophyll content of 0.5 ml samples was determined as before [11].

Preparation of intact chloroplasts. Spinach leaves (20 g) were homogenized in 20 ml 40 mM Tris–HCl buffer (pH 7.4) and the homogenate was filtered through a double layer of muslin. After centrifuging the filtrate, the supernatant obtained was layered over a discontinuous sucrose density gradient as previously described [11]. Intact chloroplast were obtained from the interface between the 41% and the 50% sucrose solutions.

Subfractionation of chloroplasts. The suspension of intact chloroplasts in 41% (w/v) sucrose was diluted with 40 mM Tris–HCl buffer (pH 7.4) to a sucrose concn of 15% (w/v). The diluted suspension was stirred for 30 min at 4° then centrifuged at 2000 g for 10 min. Samples (10 ml) of the supernatant were layered over a discontinuous sucrose gradient composed of 0.6 M (6 ml), 0.93 M (6 ml), 1.2 M (7 ml), and 1.5 M (7 ml) sucrose, in a polyallomer ultracentrifuge tube (38.5 ml capacity). The tubes were centrifuged at 105 000 g for 90 min using a 60 Ti rotor (Beckman Ltd). After centrifugation, fractions (0.5 ml) were removed and assayed for marker enzymes, protein, chlorophyll and cyclic nucleotide phosphodiesterase.

Solubilization of microsome membranes. A purified microsomal fraction (20 ml), obtained by zonal centrifugation as previously described [11] was extracted by stirring with 10 ml 40 mM Tris–HCl buffer (pH 7.4) containing 0.1% (w/v) sodium deoxycholate. The extract was centrifuged at 20 000 g for 60 min at 2°. The sediment and supernatant were assayed for protein, RNA and cyclic nucleotide phosphodiesterase activity.

Purification of cyclic nucleotide phosphodiesterase from intact chloroplasts and from the microsomal fractions. Phosphodiesterases 1_m and 1_c were prepared, as previously described [11], from the intact chloroplast and microsomal extracts, respectively. They were further purified by polyacrylamide gel electrophoresis in a discontinuous Tris–glycine buffer system [20]. After pre-electrophoresis of the stacking gel, protein samples (100 μg) in 5% (w/v) sucrose (50 μl) were applied. To each gel, 5 μl of tracking dye

soln (0.05%, w/v, bromophenol blue in gel buffer) was also added. A constant voltage of 200 V was applied for 2 hr; current was 3 mA/gel. Proteins were located with Coomassie blue [17] and their mobility calculated [21]. MW determination was made on 100 μg samples of proteins using polyacrylamide gels containing sodium dodecyl sulphate [22].

Polar lipids of cyclic nucleotide phosphodiesterase activities 1_m *and* 1_c. The polar lipid components of 1_m and 1_c were extracted from these fractions over a 12 hr period using 10 vol. chloroform–methanol (2:1). The residue was re-extracted×3 with the same solvent mixture and the combined extracts were washed with 0.2 vol. of 0.1% $MgCl_2$. After separating the phases by centrifuging at 10 000 g for 10 min, the aq. phase was discarded. The lipid extract was evapd to dryness *in vacuo* at 30° and redissolved in $CHCl_3$. The volume of this extract was reduced by evapn under N_2. Aliquots (20 ml) were chromatographed by TLC in one dimension on Si gel G in $CHCl_3$–MeOH–HOAc–H_2O (170:25:25:6). The plates had been previously activated at 110° for 30 min. After drying the plates in a stream of N_2 to minimize lipid degradation, spots were located with I_2 vapour. Following evapn of I_2, the plates were oversprayed with 0.5% (w/v) ethanolic α-naphthol in conc H_2SO_4 [23] and heated to 150°. Unknown compounds were identified by comparison of chromatographic behaviour with that of authentic standards.

Estimation of nucleic acids. DNA and RNA were estimated as previously described [11].

Examination of competitive kinetics. The cyclic nucleotide phosphodiesterase activity in peak 1_c was measured in the presence of a single concn of 3′:5′-cyclic AMP and with a series of 3′:5′-cyclic GMP concns (1 μM–1 mM); this was repeated for a similar range of 3′:5′-cyclic AMP concns in the presence of a single concn of 3′:5′-cyclic GMP. Using 2′:3′-cyclic AMP and 2′:3′-cyclic GMP at the same concn range, a similar procedure was applied to the cyclic nucleotide phosphodiesterase of peak 1_m.

Effect of p*-chloromercuribenzoate.* Cyclic nucleotide phosphodiesterases 1_c and 1_m were separately incubated with *p*-chloromercuribenzoate (1 mM) for 3 min at 37°, and the enzymes assayed for activity using appropriate controls. The inhibited enzymes were then incubated with 2-mercaptoethanol for 10 min.

Dissociation of cyclic nucleotide phosphodiesterase enzyme complexes. Attempts were made to dissociate peaks 1_c and 1_m cyclic nucleotide phosphodiesterase activities, obtained by polyacrylamide gel electrophoresis, from their associated ATPase, ribonuclease, nucleotidase and acid phosphatase (peak 1_c only) activities. The chaotropic agents used were urea, low temp., and low pH.

Peak 1_m cyclic nucleotide phosphodiesterase activity, obtained by chromatography on DEAE-cellulose in 40 mM Tris–HCl buffer at pH 7.4, was dialysed against the same buffer for 12 hr with constant stirring. After dialysis, the protein sample (2 mg) was subjected to a series of five cycles of freezing and thawing and the ppt. formed was removed by centrifugation at 10 000 g for 10 min. The supernatant (50 μl) was fractionated by polyacrylamide gel electrophoresis using 5% gels. Control samples were fractionated without the freezing and thawing procedure. Gels were stained for protein or cut into 5 mm slices and eluted for 12 hr with the buffer used for the appropriate enzyme assay. Samples were assayed for cyclic nucleotide phosphodiesterase, ATPase and ribonuclease.

Urea was used to dissociate the cyclic nucleotide phosphodiesterase complex of peak 1_c. The complex was obtained by chromatography on DEAE-cellulose in 40 mM Tris–HCl buffer (pH 7.4). It was dialysed for 8 hr against H_2O with constant stirring at 4°. The nondiffusible material (2 mg protein/ml) was mixed with 0.1 M 2-mercaptoethanol and solid urea was added to a final concn of 4 M in a total vol. of 1 ml. The protein sample (1 ml) was incubated at 25° for 60 min and then diluted with 1 ml 40 mM Tris–HCl buffer at pH 6.5. The proteins in the incubate were fractionated by polyacrylamide gel electrophoresis using 7.5% gels containing 1 M urea to prevent possible reassociation of the complex. After separation, the gels were stained for protein, or cut into 5 mm slices and eluted with 40 mM Tris–HCl buffer at pH 6.5 (500 μl). Eluates were assayed for cyclic nucleotide phosphodiesterase, ATPase, nucleotidase, ribonuclease and acid phosphatase.

The cyclic nucleotide phosphodiesterase complex of peak 1_c was also dissociated using exposure to low pH. The sample was dialysed against H_2O for 8 hr at 4°. The non-diffusible material (2 mg protein/ml) was adjusted to pH 2.8 by adding 0.1 vol. of 0.1 M glycine–HCl buffer (pH 2.8). After standing for 5 min at 4°, the acidified protein soln was neutralized with 1 M Tris–HCl buffer (pH 7.4) and immediately fractionated by polyacrylamide gel electrophoresis using 5% gels. The gels were stained for protein or sliced and eluted with an appropriate buffer for 12 hr. Eluates were examined for various enzymic activities. Reassociation was demonstrated by incubating the neutralized protein sample at pH 7.4 in 1 M Tris–HCl buffer at pH 7.4 for 60 min at 37°, and then fractionated by polyacrylamide gel electrophoresis using 5% gels. For comparison, a control protein sample was similarly treated.

Hydrolysis of RNA by ribonuclease of fractions 1_m *and* 1_c. Ribonuclease activities were obtained from the complexes by anion-exchange chromatography on columns of DEAE-cellulose. High MW yeast RNA (10 mg/ml), purified by column chromatography on Sephadex G-25, was incubated with enzyme (3 mg protein) at 37° in 50 mM sodium citrate buffer (pH 5.0); the total incubation vol. was 3 ml. Samples were removed at 60 min intervals and evapd to dryness *in vacuo* at 40°. The dry residue was redissolved in H_2O (0.5 ml) and subjected to high voltage paper electrophoresis in one dimension and PC in the second. Whatman 3 MM paper (46×57 cm) was used. The PC solvent systems were (a) 2-methylpropan-2-ol–0.02 M ammonium formate (55:45) adjusted to pH 3.7 with formic acid; (b) propan-2-ol–NH_3 (sp. gr. 0.88)–H_2O (14:3:3); (c) 2-methylpropanoic acid–NH_3 (sp. gr. 0.88)–H_2O (66:1:3). HV electrophoresis was in an HOAc–formic acid–H_2O buffer (149:41:1810) at pH 2. Samples were run for 3 hr at 3 kV. Spots were located in UV light and eluted in 0.01 M HCl. Unknown nucleotides were identified by comparison of their chromatographic, electrophoretic, and spectrophotometric properties with those of authentic samples.

Hydrolysis of RNA by ribonuclease purified from peak 1_m. The ribonuclease from peak 1_m was freed from contaminating nucleotidase and cyclic nucleotide phosphodiesterase by low temp. dissociation of the multienzyme aggregate. The latter had been obtained from polyacrylamide gel electrophoresis on 5% gels. Ribonuclease was eluted from the gels using 50 mM sodium citrate buffer (pH 5.0) at 4° for 12 hr. The ribonuclease activity was incubated with purified yeast RNA and the products identified as described above. The effect of various end products on the activity of 1_m ribonuclease was examined using 1 mM solns.

Base frequency of yeast RNA. Samples (1 ml) of yeast RNA (1% w/v) were completely hydrolysed by incubating

with 10 ml 0.3 M KOH at 37° for 10 hr. After precipitating ClO_4^- as the K salt, the supernatant was decanted, evapd to dryness and redissolved in 2 ml 0.05 M HCl. The constituent nucleotides were separated and identified. Their respective amounts were determined spectrophotometrically.

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