Whitlow, M., & Teeter, M. M. (1985) *J. Biomol. Struct. Dyn.* 2, 831–848.
Winterfeld, K., & Bijl, L. H. (1948) *Justus Liebigs Ann. Chem. 516*, 107–114.
Woodward, C., Simon, I., & Tüchsen, E. (1982) *Mol. Cell. Biochem. 48*, 135–160.
Woynarowski, J. M., & Konopa, J. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 1535–1545.

Potato Tuber Cyclic-Nucleotide Phosphodiesterase: Selective Inactivation of Activity vs. Nucleoside Cyclic 3′,5′-Phosphates and Properties of the Native and Selectively Inactivated Enzyme†

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ABSTRACT: Exposure of higher plant (potato tuber) cyclic-nucleotide phosphodiesterase (cPDase, EC 3.1.4) to alkaline pH at 45 °C leads to irreversible selective loss of activity vs. 3′,5′-cAMP with full maintenance of activity vs. 2′,3′-cAMP. There is also loss of most (~80%) of the activity vs. the *p*-nitrophenyl esters of pT and Tp but retention of full activity vs. *p*-nitrophenyl phenylphosphonate and bis(*p*-nitrophenyl) phosphate. The phosphonate ester, proposed as a specific substrate for 5′-nucleotide PDases [Kelly, S. J., Dardinger, D. E., & Butler, L. G. (1975) *Biochemistry 14*, 4983–4988], is now seen to be an excellent substrate for the activity of higher plant cPDase vs. nucleoside cyclic 2′,3′-phosphates. And, in fact, the phosphonate substrate competitively inhibits hydrolysis of 2′,3′-cAMP (K_i ~ 0.3 mM) and vice versa (K_i ~ 0.05 mM). Hydrolysis of the phosphonate ester is also inhibited by 3′,5′-cAMP, but with a K_i 1–2 orders of magnitude higher. The potato enzyme, previously shown to be a tetramer, may be reversibly dissociated to the monomer, with full retention of all activities. Selective inactivation proceeds at the level of the monomer, following which reassociation to the tetramer is minimal and not accompanied by recovery of activity vs. 3′,5′-cAMP. Isoelectric focusing resolves the enzyme into five isozyme fractions, with p*I* values ranging from 6.8 to 8.1. All five fractions exhibit identical properties as regards molecular weight and substrate specifities both prior to and following selective inactivation. The foregoing findings readily account for conflicting reports on the properties of higher plant cyclic-nucleotide phosphodiesterases. Attention is drawn to some similarities with the nonconventional mammalian cyclic-nucleotide phosphodiesterase reported by Helfman and Kuo [Helfman, D. M., & Kuo, J. F. (1982) *J. Biol. Chem. 257*, 1044–1047].

Cyclic-nucleotide phosphodiesterase (cPDase)[1] has been isolated and variously characterized from a variety of higher plants (Brown & Newton, 1981). Unlike the corresponding enzymes from mammalian cells, with one important exception (Helfman & Kuo, 1982; see below), the plant enzymes exhibit broad specificity and hydrolyze both cyclic 2′,3′- and cyclic 3′,5′-phosphates of nucleosides (Vandepeute et al., 1973; Ashton et al., 1975; Shinshi et al., 1976; Zan-Kowalczewska et al., 1984). Rare exceptions include the enzyme from beans (Brown et al., 1977), reported inactive toward nucleoside cyclic 2′,3′-phosphates, and that from cultured tobacco cells, with very low activity vs. nucleoside cyclic 3′,5′-phosphates (Matsuzaki & Hashimoto, 1981), in disagreement with another report (Shinshi et al., 1976). This confusing situation has rendered difficult attempts to clarify the functional role of plant cPDases, the more so in that almost all preparations contain nucleotide pyrophosphatase activity (Zan-Kowalczewska et al., 1984).

We have recently succeeded in purifying potato tuber cPDase to near homogeneity, with only residual (~0.5%) pyrophosphatase activity (Zan-Kowalczewska et al., 1984). Apart from previously described activities, the purified enzyme also cleaved aryl esters of nucleoside 3′- and 5′-phosphates, nucleoside 5′-di- and 5′-triphosphates, aryl phosphonates, and the 2′,3′-cGMP terminal residue of a fragment of TMV RNA (Zan-Kowalczewska et al., 1984).

During the course of further attempts to resolve the nature of the activity of this enzyme against such a multitude of substrates, it was observed that storage of the purified enzyme at 4 °C at pH 7.5 led to selective loss of activity against nucleoside cyclic 3′,5′-phosphates, without affecting activity vs. nucleoside cyclic 2′,3′-phosphates. This phenomenon has now been further examined, with results that undoubtedly contribute to a better understanding of the substrate specificity of this ubiquitous enzyme.

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[1] Abbreviations: PDase, phosphodiesterase; cPDase, cyclic-nucleotide phosphodiesterase; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; nitrophenyl-pT, thymidine 5′-(*p*-nitrophenyl phosphate); Tp-nitrophenyl, thymidine 3′-(*p*-nitrophenyl phosphate); BIS, bis(*p*-nitrophenyl) phosphate; AS-BI-naphthol, 6-bromo-2-hydroxy-3-naphthoic acid 2-methoxyanilide; AS-BI-naphthyl-pT, thymidine 5′-(AS-BI-naphthyl phosphate); NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; IEF, isoelectrofocusing; PEI, poly(ethylenimine); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Experimental Procedures

Materials. Sephadex IEF and LMW protein calibration kit were from Pharmacia (Uppsala, Sweden). Ampholines were from Serva (Heidelberg, FRG). Ampholine PAG plates, 1804-101 and 1804-103, were from LKB (Bromma, Sweden). Materials for electrophoresis and Triton X-100 were from BDH (Poole, Dorset, U.K.). Thymidine 5′-(AS-BI-naphthyl phosphate) was prepared as described elsewhere (Sierakowska et al., 1978). 2′,3′-[8-^{3}H]cAMP (24.3 Ci/mmol) and 3′,5′-[8-^{3}H]cAMP (26 Ci/mmol) were from Amersham (Bucks, U.K.), and *p*-nitrophenyl phenylphosphonate was from Regis Chemical Co. (Morton Grove, IL). NEM and PMSF were from Eastman Kodak (Rochester, NY). Other substrates and Fast Garnet GBC were from Sigma (St. Louis, MO). Thin-layer chromatography made use of Merck (Darmstadt, GFR) cellulose F and PEI–cellulose F plates. All other reagents were analytical-grade commercial products. Measurement of pH was carried out with a Radiometer (Copenhagen, Denmark) PHM 22p instrument.

Enzyme. The highly purified, homogeneous cPDase previously described (Zan-Kowalczewska et al., 1984) exhibits only residual (~0.5%) pyrophosphatase activity vs. NAD$^+$. Because of its limited availability, due to the low yield of the purification procedure, most experiments were conducted with a preparation obtained following step 7 of the purification protocol and exhibiting 3–4% residual pyrophosphatase activity, referred to as *partially purified cPDase.* The findings with this preparation were checked periodically against those obtained with the highly purified enzyme and were essentially the same. In experiments involving incubation of the enzyme for more than several hours, a crystal of thymol was added to prevent possible bacterial contamination.

Most experiments were based on the use of samples that, following selective inactivation, still posessed residual (0.4–2%) activity vs. 3′,5′-cAMP and, hence, also vs. AS-BI-naphthyl-pT (Zan-Kowalczewska et al., 1984). The latter substrate furnished a sensitive procedure for location of enzymatically active bands following gel electrophoresis (Bartkiewicz et al., 1985), as described below.

Assays of enzyme activities were essentially as previously described (Zan-Kowalczewska et al., 1984), with incubation of an appropriate amount of enzyme with 5 mM substrate (unless otherwise indicated) in 0.05 mL of 0.1 M Tris–acetate buffer, pH 6, at 37 °C for 15 min. Under these conditions the reaction was linear with time and enzyme concentration. One unit of cPDase activity is the amount of enzyme hydrolyzing 1 μmol of 2′,3′-cAMP in 15 min at 37 °C.

Kinetic studies, with 2′,3′-cAMP or 3′,5′-cAMP as substrate, were performed radiochemically (Thompson et al., 1971). The 0.05-mL reaction mixture included 0.1 M Tris–acetate buffer, pH 6.0, 0.05–0.8 mM 2′,3′-cAMP plus 4 × 10^4 cpm 2′,3′-[8-^{3}H]cAMP (or 0.5–5.0 mM 3′,5′-cAMP plus 4 × 10^4 cpm 3′,5′-[8-^{3}H]cAMP), and appropriate levels of enzyme (and inhibitor), as indicated. Incubation was terminated by heating at 100 °C for 2 min. This was followed by addition of 0.15 mL of 0.1 M Tris–acetate buffer, pH 8.0, and 0.1 unit of alkaline phosphatase, incubation for 10 min at 37 °C, and then addition of 0.4 mL of water, followed by 1.0 mL of an aqueous methanolic slurry of AG 1-X2 anion-exchange resin (Thompson et al., 1977). Liberated labeled adenosine was then measured as described by Thompson et al. (1971).

With *p*-nitrophenyl phenylphosphonate as substrate (or inhibitor), the reaction mixture (0.75 mL) included 0.1 M Tris–acetate buffer, pH 6, 0.05–1.5 mM *p*-nitrophenyl phenylphosphonate, and appropriate levels of enzyme (and inhibitor) as indicated. Incubation, for 15 min at 37 °C, was terminated by addition of 0.05 mL of 1 N NaOH. Liberated *p*-nitrophenol was then measured as described by Razzell and Khorana (1961).

Polyacrylamide gel electrophoresis (PAGE) was performed at pH 9.3 according to Davis (1964) on 0.4 mm thick 7.5% gel slabs. The enzyme solution was first concentrated by lyophilization and then dialyzed for several hours against 0.01 M Tris–acetate buffer, pH 6.0, containing 0.005% Triton X-100.

SDS–PAGE was according to Laemmli (1970) additionally modified to permit detection of active forms of the enzyme (see below). The enzyme sample was treated for 2 h at 37 °C with 2.3% SDS in 0.0625 M Tris-HCl buffer, pH 6.8, and subjected to electrophoresis on 0.4 mm thick 10.5% gel slabs at 4 °C, initially at 10 mA for 15 min and subsequently at 20 mA for 3 h. Marker proteins were treated with SDS (Laemmli et al., 1970), electrophoresed as above, and stained with Coomassie blue R-250.

Localization of cPDase activity on gels (and also following IEF) was as follows. The gel was rinsed for 5–10 min twice with cold 0.2 M acetate buffer, pH 5.2, and then either reacted histochemically for cPDase activity or sectioned into 5-mm lengths and assayed against several substrates.

Histochemical reactions were performed by incubation of a gel strip in 12 mL of 0.1 M acetate buffer, pH 5.2, plus 10 mM EDTA with 1 mg of thymidine 5′-(AS-BI-naphthyl phosphate) and 1.5 mg of Fast Garnet GBC at 37 °C for up to 2 h (Sierakowska et al., 1978).

Enzyme Activity of Gel Fractions. With nitrophenyl-pT or BIS as substrates, the gel fraction was incubated in 0.1 mL of 5 mM solutions, as above. With 2′,3′-cAMP and 3′,5′-cAMP as substrates, concentrations were 5 mM in 0.2 mL, and the reaction was terminated by heating for 3 min in boiling water, followed by addition of 0.05 mL of 0.8 M Tris-HCl buffer, pH 8.0, plus 0.1 unit of alkaline phosphatase and incubation for 10 min at 37 °C. For color development, 0.2 mL of the medium overlaying the gel was withdrawn and the enzymatically liberated P_i assayed as described elsewhere (Bartkiewicz et al., 1985).

Isoelectrofocusing (IEF), preparative and analytical, was carried out with an LKB 2117 flat-bed apparatus (Multiphor), as described in LKB Application Note No. 198 (preparative) and No. 250 (analytical). The enzyme sample was first concentrated by lyophilization and dialyzed vs. 0.1 M glycine with 0.005% Triton. Preparative IEF was with 3-mm Sephadex IEF and ampholines at pH values of 6.0–8.5 for 16 h at 4 °C, initially at 560 V and 14 mA and subsequently at 1900 V and 7 mA. A paper print was then taken of the gel solution (30 s) to reveal the focused protein zones. The print was dried immediately and sprayed with a solution for histochemical localization of activities. Relevant bands were removed from the gel and eluted with 0.1 M acetate buffer, pH 5.2. The enzyme was salted out by addition of $(NH_4)_2SO_4$ to 60% saturation; the protein precipitate was collected on a Millipore 0.45-μm filter and dissolved in 0.1 M Tris-HCl buffer, pH 7.5, containing 0.02% Triton. The individual fractions were dialyzed for 2 h vs. 0.05 M Tris–acetate buffer, pH 6.0, containing 0.01% Triton and used in this form for further analysis. The pH along the gel was determined by cutting out 2 cm side strips 0.5 cm in length. Each strip was eluted into 1 mL of water and the pH determined with a Radiometer 22p instrument with a semimicro glass electrode.

Sucrose density gradient centrifugation was performed as previously described (Zan-Kowalczewska et al., 1984).

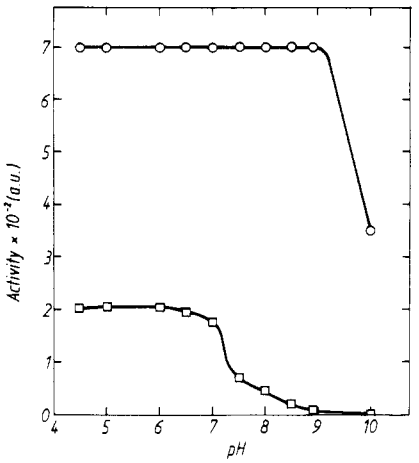

FIGURE 1: Influence of pH on activities of purified potato tuber cPDase vs. 3′,5′-cAMP (□) and 2′,3′-cAMP (○), following incubation at 45 °C for 18 h in 0.1 M buffers (acetate, pH 4.5–6; Tris-HCl, pH 7–9; glycine, pH 10). Controls were identical samples at each pH stored at −20 °C. Activities are in micromoles of substrate hydrolyzed in 15 min at 37 °C. Identical results were obtained with cytidine or deoxycytidine cyclic phosphates.

Hydrolysis products of 2′,3′-cAMP were determined chromatograpically on Merck cellulose F plates, as described by Helfman and Kuo (1982), and of 3′,5′-cAMP with the use of the ^{3}H-labeled compound on Merck PEI–cellulose F plates according to Ashton and Polya (1975).

Selective Inactivation of Activity vs. 3′,5′-cAMP. Optimal conditions were as follows: the enzyme sample was dialyzed vs. 0.1 M Tris-HCl buffer, pH 8.8, containing 0.01% Triton for 2 h at 4 °C. Enzyme activities were monitored, and the solution was divided into two aliquots. One was stored at −18 °C as a control; the other was kept at 45 °C for periods of up to 18–30 h. An additional control consisted of a sample dialyzed vs. 0.1 M Tris-acetate buffer, pH 6.0, and then kept at 45 °C.

RESULTS

The starting point for this study was the unusual observation that storage at 4 °C, in 0.02 M Tris-HCl buffer, pH 7.5, of a preparation of potato tuber cPDase established as homogeneous by various criteria (Zan-Kowalczewska et al., 1984) led to a slow time-dependent decrease in activity vs. 3′,5′-cAMP (and nitrophenyl-pT, see below) with full maintenance of activity toward 2′,3′-cAMP.

Selective Inactivation of cPDase. Storage of the enzyme solution at pH values <7 at temperatures up to 37 °C for 24 h or heating at 55 °C for 1 h, or at 45 °C at pH 5 overnight, did not affect activities against either of the cyclic phosphates. These activities were also unaffected by heating for 2 h at 37 °C, or 30 min at 45 °C in 0.07 M Tris-HCl buffer, pH 6.8, in the presence of 2.3% SDS. The latter observation was profited from by following histochemically the migration of the enzyme during SDS–PAGE (see Experimental Procedures and below).

A detailed examination of the effects of pH and temperature then demonstrated that approximately optimal conditions leading to virtually total loss of activity vs. 3′,5′-cAMP, without affecting activity vs. 2′,3′-cAMP, occurred after about 18-h incubation at 45 °C in 0.1 M Tris-HCl buffer, pH 8.8. As may be seen from Figure 1, the extent of selective loss of activity vs. 3′,5′-cAMP increased with pH in the range 7.5–8.8. At more alkaline pH, activity vs. 2′,3′-cAMP also decreased. These alkaline-induced losses in activity were not regained on reverting to pH 5.

Table I: Changes in Relative Rates of Hydrolysis of Various Substrates of Potato Tuber cPDase, following 90% Selective Inactivation of Activity vs. 3′,5′-cAMP by Heating at 45 °C, pH 8.8, for 18 h[a]

	native enzyme		selectively inactivated enzyme[b]	
substrate	rate (%)	K_m (mM)	rate (%)	K_m (mM)
2′,3′-cAMP	100	0.4	100	0.4
3′,5′-cAMP	23	1.5	2	1.5
bis(*p*-nitrophenyl) phosphate	150	0.4	105[c]	0.4
nitrophenyl-pT	30	0.4	6	0.4
Tp-nitrophenyl	30	0.4	5	
p-nitrophenyl phenylphosphonate	104	0.2	100	2.0
AS-BI-naphthyl-pT	6		0.6	
NAD^+	4.4		0[d]	

[a] This treatment did not affect activity vs. 2′,5′-cAMP, taken as 100. The native enzyme preparation was that previously reported, following the seventh purifiation step, containing residual activity toward NAD^+ (4%). [b] Level of activity vs. 3′,5′-cAMP reduced from 23 to 2% of that vs. 2′,3′-cAMP. [c] Reduction in rate is due to liquidation of residual nucleotide pyrophosphatase activity. [d] This testifies to complete inactivation of nucleotide pyrophosphatase activity.

Like most cyclic nucleotide phosphodiesterases from higher plants (Ashton & Polya, 1975; Shinshi et al., 1976; Matsuzaki & Hashimoto, 1981), the homogenous potato enzyme exhibits activities toward a variety of substrates (Zan-Kowalczewska et al., 1984). Table I presents the relative activities against various substrates of the partially purified native enzyme, and of a sample subjected to selective inactivation at 45 °C and pH 8.8 for 18 h, so that there is still residual activity vs. 3′,5′-cAMP (~2%, as compared to 23% for the native enzyme, relative to activity vs. 2′,3′-cAMP). The disappearance of the low activity vs. NAD^+ [due to contamination with nucleotide pyrophosphatase activity (see Enzyme)] in the alkali-treated preparation is readily accounted for by the observed lability of nucleotide pyrophosphatase under these conditions [cf. Bartkiewicz et al. (1985)]. The partial decrease in activity toward BIS (also a substrate for potato nucleotide pyrophosphatase) is due to the same cause. By contrast, the marked diminution in activities vs. the *p*-nitrophenyl esters of pT and Tp and the AS-BI-naphthyl phosphate ester of pT parallels the drop in activity toward 3′,5′-cAMP. The unchanged activity toward *p*-nitrophenyl phenylphosphonate (Table I) was quite unexpected and is discussed separately, below.

The foregoing experiments, in which reduction of activity vs. 3′,5′-cAMP was incomplete, made possible determination of the K_m value for this residual activity and comparison with the K_m values for both cyclic phosphates in the native preparation. The values obtained (Table I), 1.5 mM for 3′,5′-cAMP and 0.4 for 2′,3′-cAMP, were identical in both cases to those previously reported for the native enzyme (Zan-Kowalczewska et al., 1984).

Selective inactivation did not lead to any changes in the pH optima for activity vs. 2′,3′-cAMP or the residual activity toward 3′,5′-cAMP. By contrast, for the phosphonate ester, the broad pH optimum of 6.5–8 for the native enzyme was shifted to about pH 4 for the selectively inactivated enzyme (see Figure 2).

Chromatographic analysis of the products of hydrolysis of 3′,5′-cAMP demonstrated a 4:1 mixture of 3′-AMP and 5′-AMP, respectively. With 2′,3′-cAMP as substrate, the only product of hydrolysis was 3′-AMP.

Substrate and Inhibitor Properties of p-Nitrophenyl Phenylphosphonate. This synthetic compound has been described

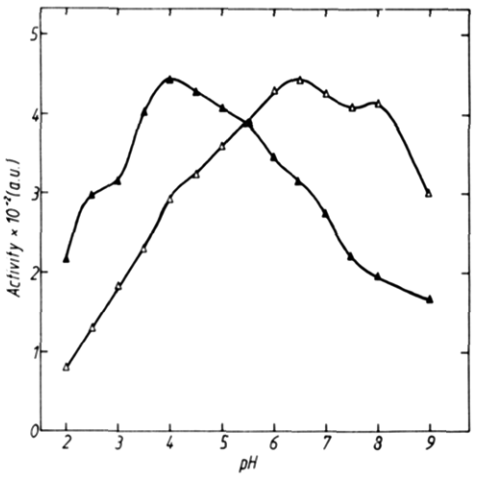

FIGURE 2: pH dependence of activity of native (△) and alkali-treated (▲) potato cPDase vs. *p*-nitrophenyl phenylphosphonate.

Table II: Inhibition Constants for Hydrolysis of Key Substrates by Native and Selectively Inactivated Potato Tuber cPDase

substrate	inhibitor	K_i (mM) native enzyme	K_i (mM) selectively inactivated enzyme[a]
2′,3′-cAMP	P_i	0.73 ± 0.12	0.15 ± 0.02
	p-nitrophenyl phenylphosphonate	0.27 ± 0.04	0.32 ± 0.04
	3′,5′-cAMP	8.0 ± 0.7	5.0 ● 0.6
p-nitrophenyl phenyl-phosphonate	P_i	0.3 ● 0.04	0.2 ● 0.04
	2′,3′-cAMP	0.05 ● 0.02	0.09 ● 0.03
	3′,5′-cAMP	6.0 ± 1.2	1.4 ± 0.4
3′,5′-cAMP[b]	P_i	0.04 ± 0.01	0.05 ± 0.01

[a] Level of activity vs. 3′,5′-cAMP reduced from 23 to 2% of activity vs. 2′,3′-cAMP. [b] Inhibition by 2′,3′-cAMP and *p*-nitrophenyl phenylphosphonate not measured because they are rapidly hydrolyzed substrates.

by Kelly et al. (1975, 1977) as a substrate specific for 5′-nucleotide PDases. We have previously noted that it is hydrolyzed at pH 6 by potato cPDase much more rapidly than 3′,5′-cAMP and, in fact, at a rate comparable to that for 2′,3′-cAMP (Zan-Kowalczewska et al., 1984). We now find that, at 5 mM, its V_{max} at pH 6 (360 μmol min^{-1} mg^{-1}) is identical with that for 2′,3′-cAMP and that the K_m for the phosphonate is 0.2 mM (previously erroneously reported as 0.7 mM), similar to the K_m of 0.4 mM for 2′,3′-cAMP (Table I). Furthermore, the selectively inactivated enzyme exhibits virtually unchanged activity toward this substrate, but with a 10-fold higher K_m (Table I). In line with this, the observed rate of hydrolysis of a 5 mM solution is lower than V_{max}. This explains why, despite the 2-fold increase in V_{max} following selective inactivation, the rates of hydrolysis of a 5 mM solution are similar to those of the native enzyme and the selectively inactivated one.

Kinetic studies revealed that 2′,3′-cAMP competitively inhibits hydrolysis of the phosphonate ester (K_i = 0.05 mM) and vice versa (K_i = 0.27 mM) and that these values are essentially unchanged with the selectively inactivated enzyme (Table II). This mutual competitive inhibition between 2′,3′-cAMP and the phosphonate ester suggests that both are bound at a common site. Hydrolysis of the phosphonate, and of 2′,3′-cAMP, was also competitively inhibited by 3′,5′-cAMP but with appreciably higher K_i values, 6 mM and 8 mM, respectively. Since the phosphonate ester is a substrate for potato cPDase activity vs. 2′,3′-cAMP but not 3′,5′-cAMP, it appeared of interest to examine susceptibility of the phosphonate

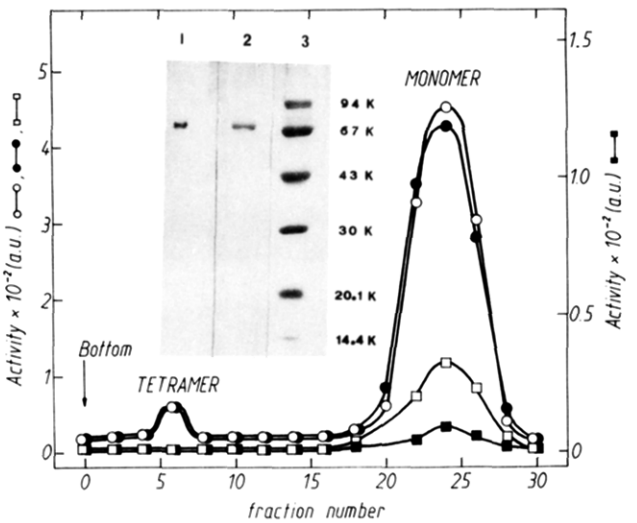

FIGURE 3: Sedimentation in a saccharose gradient (5–15%, pH 7.8) of native (○, □) and selectively inactivated (●, ■) potato tuber cPDase (10 units in terms of activity vs. 2′,3′-cAMP deposited on gradient). Fractions of 7 drops were collected and activities assayed vs. 2′,3′-cAMP (○, ●) and 3′,5′-cAMP (□, ■) as micromoles hydrolyzed in 15 min at 37 °C. (Insert) Modified SDS–polyacrylamide gel electrophoresis of selectively inactivated potato cPDase (14 units, 2.5 μg): (lane 1) enzyme localized histochemically with AS-BI-naphthyl-pT as substrate; (lane 2) enzyme localized by Coomassie blue staining; (lane 3) protein markers stained with Coomassie blue.

to an enzyme such as rat brain 2′,3′-cPDase. With a rat brain extract as described by Wells and Sprinkle (1981), no activity could be detected against the phosphonate.

Competition by P_i. It is of interest, in relation to the foregoing, that hydrolysis of the phosphonate and of 2′,3′-cAMP was competitively inhibited by P_i with comparable K_i values, 0.3 mM and 0.7 mM. By contrast, hydrolysis of 3′,5′-cAMP was inhibited by P_i with a K_i = 0.04 mM (Table II).

Role of Enzyme Monomer. It was previously shown that the native form of potato tuber cPDase is a tetramer, which readily dissociates to the monomer in 5 M urea at neutral pH or in aqueous alkaline medium above pH 8.5 at 0 °C. Subsequent acidification to pH 5 led to ~80% re-formation of the tetramer. Furthermore, dissociation and reaggregation at 0 °C were not accompanied by any changes in substrate properties (Zan-Kowalczewska et al., 1984).

We have now found that selective loss of activity vs. 3′,5′-cAMP does not occur in the presence of 5 M urea, even at pH 7.5 or 8.8 at 4 °C, during the period (~2 h) required for complete conversion to the monomer. Since, as pointed out above, selective loss of activity vs. 3′,5′-cAMP on heating at alkaline pH is not reversed on subsequent acidification to pH 5, it follows that selective inactivation occurs at the level of the monomer. In accordance with this, it was found that, following selective inactivation, reaggregation to the tetramer on exposure to pH 5 is reduced to less than 20%. Furthermore, when the fully active monomeric form of the enzyme and a selectively inactivated sample were each subjected to centrifugation in a 10–20% saccharose gradient at pH 7.8, both sedimented at identical locations in the gradient (Figure 3).

Similarly, an alkaline-treated enzyme sample, when subjected to modified SDS–PAGE (see Experimental Procedures), showed up as a single band, both chromatographically by Coomassie blue staining and histochemically with the use of AS-BI-naphthyl-pT as substrate (insert of Figure 3). This band coincided with that of the fully active homogeneous monomer (Zan-Kowalczewska et al., 1984).

Although potato tubers are known to contain very low, or negligible, levels of proteolytic activity (Laskowski & Katō,

Table III: Characteristics of Fractions of Potato cPDase Obtained by Preparative Isoelectrofocusing

		eluted activity vs.			
		2′,3′-cAMP		3′,5′-cAMP	
fraction no.	p*I*	%	units	%	units
1	6.8	15	3.2	20	0.11
2	7.0	21	4.4	18	0.10
3	7.4	28	6.0	21	0.12
4	7.6	23	4.8	21	0.12
5	8.1	13	2.8	18	0.10

1980), several experiments on the time course of selective inactivation of activity vs. 3′,5′-cAMP were carried out in the presence of protease inhibitors (NEM and PMSF, both active as inhibitors at pH 8.8) at concentrations of 20 mM. There were no detectable effect on the course or level of selective inactivation, excluding possible intervention of protease activity.

Role of Heterocyclic Base. Although, for convenience, 2′,3′- and 3′,5′-cAMP were used as standard substrates throughout this investigation, we have found that change of the base from a purine to pyrimidine, or of the sugar from ribose to deoxyribose, all of which are substrates (Zan-Kowalczewska et al., 1984), gave identical results on selective inactivation.

Isoelectric Focusing. A sample of 50 units of the partially purified enzyme, subjected to preparative isoelectric focusing, gave five enzymatically active fractions with varying isoelectric points (Table III). All fractions exhibited similar activity vs. 2′,3′-cAMP and 3′,5′-cAMP (Table III), as well as against BIS, nitrophenyl-pT, and AS-BI-naphthyl-pT (Figure 4). Furthermore, all fractions exhibited comparable distributions of activities toward the different substrates. In particular, isoelectric focusing (pH 6.5–8) led to a 10-fold decrease in activity vs. 3′,5′-cAMP, relative to that against 2′,3′-cAMP, and a corresponding decrease in activity vs. nitrophenyl-pT, due to unavoidable warming at alkaline pH.

Analytical IEF, in the pH range 5.5–8.5 with myoglobin as standard (p*I* = 7.2, as against 7.3 reported by others), was then employed to determine more accurately the isoelectric points of the fractions revealed by preparative IEF. Runs were made with the native enzyme and with a selectively inactivated sample, following which the control gel lane was sliced into 0.5 × 1 cm strips, the pH of individual strips measured, and the resulting pH scale applied against the location of isozyme fractions determined with the aid of AS-BI-naphthyl-pT. The resulting p*I* values are in good agreement with those obtained by preparative IEF (Table III). A further point of interest is that each fraction (of both native and selectively inactivated enzyme) consisted of two barely distinguishable bands with p*I* values differing by 0.05–0.1 pH unit. Identical results were obtained with a high purified enzyme preparation, and fractionation of the isozymes is most likely due to the glycoprotein nature of the enzyme (see Discussion).

The foregoing findings demonstrated that both the untreated enzyme and the selectively inactivated one each contain the same five fractions. Hence, selective loss of activity vs. 3′,5′-cAMP does not lead to disappearance or appearance of any isozyme fraction with activities other than those exhibited by the native enzyme.

SDS–PAGE Analysis. Analysis of fractions 1–5, obtained by preparative IEF, by means of SDS–gel electrophoresis demonstrated that each gave a single band with activity vs. AS-BI-naphthyl-pT, the location of which on the gel corresponded to that of the enzyme monomer (Zan-Kowalczewska et al., 1984).

Further analysis of the same fractions by electrophoresis at pH 9.3 and location of the bands with the aid of AS-BI-naphthyl-pT revealed them with differing and decreasing, reciprocally proportional to their p*I* values, migration rates (Figure 4). Analogous electrophoresis of a partially purified preparation and of one with selective inactivation of activity vs. 3′,5′-cAMP led in each case to bands corresponding to those of fractions 1–5.

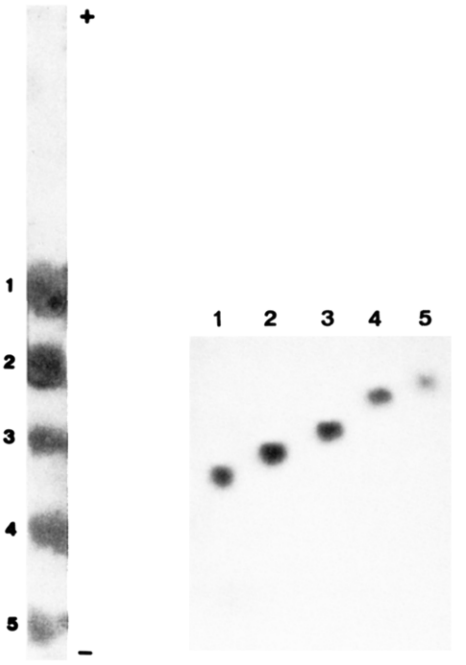

FIGURE 4: Fractionation of potato cPDase by preparative isoelectrofocusing (left frame; p*I* values: 1, pH 6.8; 2, pH 7.05; 3, pH 7.2; 4, pH 7.4; 5, pH 8.1) and by polyacrylamide gel electrophoresis at pH 9.3 of the isozyme fractions (right frame; lanes 1 and 5, 0.8 unit; lanes 2–4, 1.6 units). Localization of fractions with AS-BI-naphthyl-pT.

As pointed out above, the use of selectively inactivated enzyme that still exhibited residual activity vs. 3′,5′-cAMP and, hence, also vs. AS-BI-naphthyl-pT made it possible to use the latter substrate for sensitive histochemical localization of the enzyme bands. However, this does not exclude the possible existence of an enzyme fraction inactive against the foregoing substrates and active only vs. 2′,3′-cAMP and BIS. Hence, following electrophoresis of the native and alkali-inactivated enzyme samples, the gel lanes were subdivided into 0.5 × 0.5 cm fractions. These were cut out and assayed individually for activities vs. 2′,3′-cAMP, BIS, and nitrophenyl-pT. The results indicated that the activities of each eluate against the four substrates corresponded to that vs. AS-BI-naphthyl-pT. There was no instance of activity against only one of the foregoing substrates.

Discussion

The ability of potato tuber cPDase to undergo selective loss of activity vs. 3′,5′-cAMP, with full maintenance of activity vs. 2′,3′-cAMP, apart from its intrinsic interest, is of obvious relevance to the difference in reported behavior of cPDases from higher plants [e.g., Ashton and Polya (1975), Brown and Newton (1981), Vandepeute et al. (1973), and Zan-Kowalczewska et al. (1984)]. This is best illustrated by the conflicting reports of Shinshi et al. (1978) and Matsuzaki and Hashimoto (1981) on the properties of highly purified cPDase from cultured tobacco cells, for which our results furnish a logical interpretation.

The preparation of Shinshi et al. (1978) exhibited an M_r of 280 000 by gel filtration and a subunit M_r of 75 000 on SDS–PAGE. It was active vs. both 2′,3′-cAMP and 3′,5′-

cAMP, as well as NAD^+. The latter finding points to contamination with nucleotide pyrophosphatase (Zan-Kowalczewska et al., 1984), and the apprent homogeneity of the preparation is due simply to the fact that cPDase and nucleotide pyrophosphatase of higher plants are both tetramers with similar molecular weight values and charge, while each dissociates to monomeric units with the same mobility under standard conditions of SDS–PAGE (Zan-Kowalczewska et al., 1984).

By contrast, the preparation of Matsuzaki and Hashimoto (1981) is a monomer with a molecular weight, by gel filtration and SDS–PAGE, of about 63 000 (actually consisting of two components with molecular weight values of 65 000 and 60 000 and with differing p*I* values on gel electrophoresis of 7.2 and 7.3), with no activity vs. NAD^+, hence not contaminated with nucleotide pyrophosphatase activity. Examination of the purification procedure showed that the final step was preparative gel electrophoresis for 30 h at pH 9.3. Under such conditions, in light of our findings, one would expect only the monomeric form, as reported. Furthermore, even slight warming during such prolonged electrophoresis at alkaline pH accounts for total loss of activity vs. NAD^+ (Bartkiewicz et al., 1985) and to selective loss of activity vs. 3′,5′-cAMP relative to 2′,3′-cAMP. Examination of Table III of Matsuzaki and Hashimoto (1981) shows this is indeed the case. In fact, the relative activities of their preparation toward several key substrates exhibit a striking parallel to those of our alkaline-treated enzyme. Furthermore, their enzyme, like ours (Zan-Kowalczewska et al., 1984), exhibits little dependence of activity vs. cyclic 3′,5′-phosphates on the nature of the base and a marked dependence on the base in the case of cyclic 2′,3′-phosphates.

The thermal alkali-induced selective inactivation observed in this study is by no means unique. One recent such example is *Escherichia coli* DNA polymerase I, which, under conditions almost identical with ours (pH 8.5 at 42 °C), loses 95% of its exonuclease activity, with retention of more than 50% of its polymerase activity, and resulting in lower fidelity of replication of the latter (Lecomate & Doubleday, 1983).

As shown above, both the native and selectively inactivated forms of purified potato cPDase are separated by IEF into five isozyme fractions with similar relative activities toward *all* substrates examined. These fractions do not differ in mobility in SDS–PAGE, each giving a band corresponding to the monomer of the highly purified enzyme, and on electrophoresis at pH 9.3 exhibit decreasing mobilities with p*I* values of 6.8–8.1. We have shown that both the potato enzyme (unpublished) and that from tobacco cells (Shinshi & Katō, 1981) are glycoproteins, and this probably accounts for the separation by IEF into five fractions with different p*I* values (Gibbons, 1972; Lin, 1975). A more accurate description of the differences in charge among the different fractions must await improvements in the purification procedure (now under way) to give a higher yield of enzyme in order to determine amino acid compositions and the nature of the sugar components.

The present findings may be of direct relevance to potato as well as other plant cPDases. Our enzyme resembles in a number of respects a nonconventional mammalian cPDase, so-called cyclic-CMP phosphodiesterase from pig liver (Helfman & Kuo, 1982), which hydrolyzes both 2′,3′- and 3′,5′-cyclic nucleotides, probably at a single active site. It differs from ours in that 2′,3′-cyclic nucleotides are cleaved to a mixture of nucleoside 2′- and 3′-phosphates, while 3′,5′-cyclic nucleotides are converted uniquely to nucleoside 5′-phosphates. The pig liver enzyme, like ours, also hydrolyzes *p*-nitrophenyl phenylphosphonate, and the latter, as with our enzyme, inhibits hydrolysis of 2′,3′- and 3′,5′-cyclic nucleotides, although no quantitative data were given. It appears likely that the pig liver enzyme will attack nitrophenyl-pT and Tp-nitrophenyl, and it would be of obvious interest to also examine its activity vs. NAD^+ and BIS, as well as the effect of mild heating at alkaline pH.

The high activity of potato tuber cPDase vs. *p*-nitrophenyl phenylphosphonate calls for special comment. This interesting compound was proposed by Kelly et al. (1975, 1977) as a specific substrate for 5′-nucleotide PDases, capable of distinguishing among different types of enzymes. Our highly purified enzyme, which hydrolyzes 2′,3′-cAMP exclusively to 3′-AMP and 3′,5′-cAMP largely to 3′-AMP (actually 80% 3′-AMP and 20% 5′-AMP), exhibits very high activity against this substrate (Table I). More significantly, this high level of activity is maintained following selective liquidation of activity vs. 3′,5′-cAMP. Bearing in mind also the mutual competitive inhibition between the phosphonate ester and 2′,3′-cAMP, it is clear that the phosphonate ester cannot be considered specific for 5′-nucleotide PDases. It should also be noted that the phosphonate ester of Kelly et al. (1975, 1977) is a substrate for enzymes that hydrolyze the pyrophosphate linkage in NAD^+ (Bartkiewicz et al., 1984) and are at the same time exonucleases. Neither our purified enzyme nor the pig liver cyclic CMP PDase of Helfman and Kuo (1982) hydrolyzes NAD^+ or single-stranded RNA. Nonetheless, we have found, in agreement with Kelly et al. (1975), that the phosphonate ester is indeed a very convenient substrate for assaying overall levels of PDase activity.

Finally, attention should be drawn to an additional difference in specifities between potato (and presumably other higher plant) cPDase and conventional mammalian cPDases. We have elsewhere shown that the cyclic 3′,5′-phosphates of 2′,3′-seconucleosides (in which the 2′,3′ bond has been cleaved by periodate oxidation and borohydride reduction) are resistant to beef heart cPDase but are substrates for potato cPDase (Lassota et al., 1986). We have since found, as anticipated, that it is the activity of potato cPDase vs. nucleoside cyclic 3′,5′-phosphates that is active against the seconucleoside cyclic 3′,5′-phosphates, since this activity disappears following selective inactivation of the enzyme.

Registry No. P_i, 14265-44-2; cPDase, 9037-18-7; 2′,3′-cAMP, 634-01-5; 3′,5′-cAMP, 60-92-4; BIS, 645-15-8; NAD^+, 53-84-9; AS-BI-naphthyl-pT, 69295-52-9; nitrophenyl-pT, 2304-08-7; Tp-nitrophenyl, 16562-50-8; *p*-$O_2NC_6H_4OP(O)OPh$, 57072-35-2.

REFERENCES

Ashton, A. R., & Polya, G. M. (1975) *Biochem. J. 149*, 329–339.

Bartkiewicz, M., Sierakowska, H., & Shugar, D. (1984) *Eur. J. Biochem. 143*, 419–426.

Bartkiewicz, M., Grzybowska, E., Sierakowska, H., & Shugar, D. (1985) *Biochim. Biophys. Acta 830*, 313–319.

Brown, E. G., & Newton, R. P. (1981) *Phytochemistry 20*, 2453–2463.

Brown, E. G., Al-Najafi, T., & Newton, R. P. (1977) *Phytochemistry 16*, 1333–1337.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.

Gibbons, R. A. (1972) in *Glycoproteins* (Gottschalk, A., Ed.) Part A, pp 31–140, Elsevier, Amsterdam.

Helfman, D. M., & Kuo, J. F. (1982) *J. Biol. Chem. 257*, 1044–1047.

Kelly, S. J., & Butler, L. G. (1977) *Biochemistry 16*, 1102–1104.

Kelly, S. J., Dardinger, D. E., & Butler, L. G. (1975) *Biochemistry 14*, 4983–4988.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laskowski, M., Jr., & Katō, J. (1980) *Annu. Rev. Biochem. 49*, 593–628.
Lassota, P., Kazimierczuk, Z., Zan-Kowalczewska, M., & Shugar, D. (1986) *Biochem. Biophys. Res. Commun. 137*, 453–460.
Lecomate, J., & Doubleday, O. P. (1983) *Nucleic Acids Res. 11*, 7505–7515.
Lin, C. W. (1975) in *Isozymes* (Markert, C. L., Ed.) pp 637–651, Academic, New York.
Linneweaver, H., & Burk, D. (1934) *J. Am. Chem. Soc. 56*, 658–666.
Matsuzaki, H., & Hashimoto, Y. (1981) *Agric. Biol. Chem. 45*, 1317–1325.
Razzell, W. E., & Khorana, H. G. (1961) *J. Biol. Chem. 236*, 1144–1149.
Shinshi, H., & Katō, K. (1978) *Biochim. Biophys. Acta 524*, 357–361.
Shinshi, H., & Katō, K. (1981) *Phytochemistry 20*, 816–818.
Shinshi, H., Miwa, M., Katō, K., Noguchi, M., Matsushima, T., & Sugimura, T. (1976) *Biochemistry 15*, 2185–2190.
Shinshi, H., Katō, K., Miwa, M., Matsushima, T., Noguchi, M., & Sugimura, T. (1977) *Biochim. Biophys. Acta 495*, 71–76.
Sierakowska, H., Gahan, P. B., & Dawson, A. L. (1978) *Histochem. J. 10*, 679–693.
Thompson, W. J., & Appleman, M. M. (1971) *Biochemistry 10*, 311–316.
Thompson, W. J., Ross, C. P., Pledger, W. J., Strada, S. J., Banner, R. L., & Hersh, E. M. (1977) *J. Biol. Chem. 251*, 4922–4929.
Vandepeute, J., Huffaker, R. C., & Alvarez, R. (1973) *Plant Physiol. 52*, 278–282.
Weber, K. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wells, M. R., & Sprinkle, T. J. (1981) *J Neurochem. 36*, 633–639.
Zan-Kowalczewska, M., Bartkiewicz, M., Sierakowska, H., & Shugar, D. (1984) *Biochim. Biophys. Acta 788*, 62–73.

Mechanistic Studies on the Phytylation and Methylation Steps in Bacteriochlorophyll *a* Biosynthesis: An Application of the ^{18}O-Induced Isotope Effect in ^{13}C NMR†

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ABSTRACT: The high-resolution ^{13}C NMR spectrum of bacteriochlorophyll *a* biosynthesized from [1-^{13}C,1,1,4-$^{18}O_3$]-5-aminolevulinic acid by growing cells of *Rhodopseudomonas sphaeroides* has shown both the C-17[3] and C-13[3] resonances consist of three additional components upfield shifted from the —^{16}O—^{13}C=^{16}O resonance. By comparison with the ^{13}C NMR spectrum obtained for phytyl acetate containing ^{13}C and ^{18}O selectively in the ester linkage, these components have been identified as the bridge (—^{18}O—^{13}C=^{16}O), nonbridge (—^{16}O—^{13}C=^{18}O), and dual-labeled (—^{18}O—^{13}C=^{18}O) isotopomers. These results have been interpreted to suggest that both the ester bonds of bacteriochlorophyll *a* are produced by a carboxy–alkyl transfer process.

A common characteristic of all known chlorophylls is the esterification of the D-ring propionate carboxy group at C-17[3] with a long-chain alcohol moiety (for example, see structure III, Figure 1) (Akhtar & Jordan, 1979). The precise nature of this alcohol varies considerably, and it has been shown to be predominantly *all-trans*-farnesol (in association with smaller quantities of other long-chain alcohols) for bacteriochlorophylls *c* and *d* (Rapoport & Hamlow, 1961; Caple et al., 1978), geranylgeraniol for bacteriochlorophyll *a* from *Rhodospirillum rubrum* (Katz et al., 1976; Walter et al., 1979), tetrahydrogeranylgeraniol for bacteriochlorophyll *b* from *Ectothiorhodospira halochloris* (Steiner et al., 1981), and phytol for bacteriochlorophyll *a* from *Rhodopseudomonas sphaeroides* (Brockman, 1971) and for the plant chlorophylls (Brockman, 1971; Fischer & Wenderoth, 1939).

Historically, investigations on the esterification process at C-17[3] can be traced back to Willstätter and Stoll (1910), who exploited an earlier discovery of Borodin (1882), noting the formation of ethyl chlorophyllide from ethanol extracts of green leaves, and established that the former was produced by the action of chlorophyllase, an enzyme present in the leaves, on chlorophyll *a*. The enzyme, which was solubilized in 40% aqueous acetone and was functional in this mixture, catalyzed the hydrolysis of chlorophyll *a* to chlorophyllide *a* and phytol in a reversible process and participated in a variety of transesterification reactions. Since the equilibrium constant for the former reaction in aqueous solution favored chlorophyll hydrolysis, the major biological function of chlorophyllase was assumed to be in chlorophyll degradation. However, following the identification by Granick and colleagues that, in plants, chlorophyllide *a* was the penultimate tetrapyrrole intermediate which merely required esterification for conversion to chlorophyll *a*, chlorophyllase acquired a new significance—that of a role in chlorophyll synthesis via a reversal of the hydrolytic reaction [Granick, 1950; for a review see Granick and Beale (1978)].

Support for this view came from the experiments of Holden (1961), which showed that the activity of chlorophyllase

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