Registry No. Protein kinase, 9026-43-1; BD-S, 67701-59-1; Kemptide, 65189-71-1; Arg-Gly-Tyr-Ala-Leu-Gly, 59587-24-5; glycogen synthase, 9014-56-6.

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Activation of Bovine Brain Calmodulin-Dependent Protein Phosphatase by Limited Trypsinization†

E. Ann Tallant and Wai Yiu Cheung*

ABSTRACT: A calmodulin-dependent protein phosphatase isolated from bovine brain [Tallant, E. A., & Cheung, W. Y. (1983) *Biochemistry 22*, 3630–3635] is stimulated by limited trypsinization to the same activity level as that by calmodulin. Prolonged trypsinization caused gradual loss of phosphatase activity, a process retarded in the presence of Ca^{2+}, and even more in the presence of calmodulin. Trypsinized phosphatase, when fully activated, had a molecular weight of 60 000 and was composed of two protein species of 43 000 and 16 000 daltons. Trypsinization decreased the K_m of phosphatase for casein from 10.8 to 1.2 μM and increased the V_{max} from 4.9 to 30.9 nmol (mg of protein)$^{-1}$ min^{-1}. The proteolyzed enzyme was insensitive to calmodulin and did not bind to a calmodulin–Sepharose affinity column. It was, however, stimulated by Ca^{2+}, requiring 0.4 μM Ca^{2+} for half-maximal activation. Both native and trypsinized phosphatase were stimulated by Mn^{2+} to a level considerably higher than that by Ca^{2+}.

Calmodulin-dependent protein phosphatase, a major calmodulin-binding protein in bovine brain extracts, catalyzes the dephosphorylation of a variety of substrates, including the α subunit of phosphorylase kinase, inhibitor 1, casein, and histone (Stewart et al., 1982; Yang et al., 1982). The enzyme, referred to as CaM-BP_{80} (Wallace et al., 1980) or calcineurin (Klee et al., 1979) before its identification as a phosphatase, has a molecular weight (M_r) of 80 000 and is composed of two hetero subunits (Sharma et al., 1979; Wallace et al., 1979); subunit A (M_r 60 000) binds calmodulin (Richman & Klee, 1978), and subunit B (M_r 16 500) is itself a Ca^{2+}-binding protein (Klee et al., 1979). The enzyme is predominantly found in

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neural tissue (Wallace et al., 1980a) and has been localized at pre- and postsynaptic sites (Wood et al., 1980; Cooper et al., 1982).

Activation of a calmodulin-dependent enzyme by a protease was first noted with cyclic nucleotide phosphodiesterase of bovine brain; the stimulation was caused by proteases in snake venom, which was used as a source of 5′-nucleotidase, an ancillary enzyme in the assay of phosphodiesterase. This finding was instrumental in the discovery of a protein "activator" now known as calmodulin (Cheung, 1981). Subsequently, limited trypsinization was shown to fully stimulate phosphodiesterase, making it insensitive to calmodulin (Cheung, 1971). Other calmodulin-dependent enzymes were later found to be stimulated by limited proteolysis; they include skeletal muscle phosphorylase kinase (Depaoli-Roach et al., 1979), erythrocyte Ca^{2+}-ATPase (Carafoli et al., 1982), and myosin light chain kinase of both smooth and skeletal muscle (Walsh et al., 1982; Srivastava & Hartshorne, 1982).

The present study was undertaken to determine the effect, if any, of limited tryptic digestion on the activity of a calmodulin-dependent protein phosphatase. Our results show that limited trypsinization indeed stimulated phosphatase, in a manner similar to other calmodulin-dependent enzymes. A preliminary report has appeared (Tallant & Cheung, 1983a).

Materials and Methods

Materials

[γ-^{32}P]ATP (2 mCi/mmol), $^{14}CH_3$ methylated bovine serum albumin (0.016 mCi/mg), and $^{14}CH_3$ methylated ovalbumin (0.012 mCi/mg) were purchased from New England Nuclear, and vitamin-free casein was from ICN Nutritional Biochemicals. Sigma Chemical Co. supplied bovine serum albumin, dithiothreitol (DTT),[1] EGTA, cyanogen bromide activated Sepharose 4B, and soybean trypsin inhibitor. A calcium standard was obtained from Fisher Scientific Co.; trypsin was from Worthington. Bovine brains and hearts were generous gifts of Fineberg Packing Co. of Memphis. All other reagents were of the highest analytical grade.

Calmodulin was purified to apparent homogeneity by using fluphenazine–Sepharose (Wallace et al., 1980b) or phenyl-Sepharose affinity column chromatography (Gopalakrinshna & Anderson, 1982). The catalytic subunit of cAMP-dependent protein kinase was isolated from bovine heart according to a procedure developed for bovine liver (Sugden et al., 1976). Calmodulin-dependent protein phosphatase was purified to apparent homogeneity from bovine brain (Tallant & Cheung, 1983b).

Methods

Preparation of ^{32}P-Labeled Casein. Casein was phosphorylated with [γ-^{32}P]ATP by using the catalytic subunit of bovine heart cAMP-dependent protein kinase (Tallant & Cheung, 1983b). Casein was dephosphorylated prior to the incorporation of [^{32}P]P_i by the procedure of Reimann et al. (1971). A typical preparation of phosphorylated substrate contained 0.2–0.3 mol of bound [^{32}P]P_i per mol of casein (M_r 24 000).

Phosphatase Assay. Phosphatase activity was measured by the release of [^{32}P]P_i from ^{32}P-labeled casein. The enzyme was assayed in a reaction mixture (final volume 50 μL) containing 50 mM Tris-HCl (pH 7.0), 0.5 mM DTT, 0.1 mM $CaCl_2$, 1 mg/mL bovine serum albumin, 1.5 μM ^{32}P-labeled casein (about 40 000 cpm, calculated by the [^{32}P]phosphate concentration), and an appropriate amount of enzyme. The reaction rate was linear up to 15% phosphate released and was, therefore, kept within this limit. The ^{32}P-labeled radioactivity could be extracted into acid–molybdate, demonstrating that the radiolabeled activity represented phosphate and not phosphopeptides released by a trace of endogenous tissue proteases. Phosphatase activity is expressed as nanomoles of phosphate released per milligram of protein per minute, the background (with boiled phosphatase) having been subtracted. Each determination was duplicated.

Limited Proteolysis of Phosphatase by Trypsin. Unless otherwise indicated, phosphatase was trypsinized at 30 °C for 20 min in 50 mM Tris-HCl (pH 7.0), 0.5 mM DTT, and 0.1 mM $CaCl_2$. The ratio of phosphatase to trypsin was 100 to 1, on the basis of protein concentration. Trypsinization was terminated by the addition of a 4-fold excess of soybean trypsin inhibitor. Control or zero-time samples were treated with trypsin inhibitor prior to the addition of trypsin.

Calcium–EGTA Buffer. The level of free Ca^{2+} was determined by using a Ca^{2+}–EGTA system containing 2 mM EGTA. Prior to use, calmodulin, phosphatase and ^{32}P-labeled casein were extensively dialyzed against 50 mM Tris-HCl (pH 7.0) and 0.5 mM DTT containing 2 mM EGTA. Concentrations of Ca^{2+} were calculated according to the computer program of Perrin & Sayce (1967) by using the following logarithmic association constants: H^+ to $EGTA^{4-}$, 9.32; H^+ to $H\text{-}EGTA^{3-}$, 8.70; H^+ to H_2EGTA^{2-}, 2.66; H^+ to H_3EGTA^{1-}, 2.0; Ca^{2+} to $EGTA^{4-}$, 10.76; Ca^{2+} to $H\text{-}EGTA^{3-}$, 3.85 (Piascik et al., 1980).

Polyacrylamide Gel Electrophoresis. Discontinuous SDS–polyacrylamide gel electrophoresis was performed in a linear 7.5–15% acrylamide gradient containing 1 mM EGTA according to Laemmli (1970). Immediately before electrophoresis, samples were denatured by boiling in the presence of 1% SDS and 5% β-mercaptoethanol. Protein was stained with Coomassie brilliant blue.

Protein Determination. Protein was determined according to Lowry et al. (1951), after the proteins were precipitated with 10 volumes of cold 12.5% Cl_3CCOOH. Bovine serum albumin was used as a standard.

Results

Stimulation of Phosphatase by Trypsinization. Calmodulin-dependent protein phosphatase was treated with trypsin in the presence or absence of Ca^{2+} (Figure 1). At the times indicated, trypsinization was stopped with trypsin inhibitor, and an aliquot of the incubation mixture was assayed for phosphatase activity in the presence of EGTA, Ca^{2+}, or Ca^{2+} and calmodulin. When the enzyme was trypsinized in the presence of Ca^{2+} (panel A), its activity increased with time of trypsinization, whether assayed in EGTA (lower curve) or in Ca^{2+} (middle curve); in the presence of Ca^{2+}, phosphatase activity was 40% higher throughout the time course of trypsinization. In the presence of Ca^{2+} and calmodulin (upper curve), the enzyme activity remained essentially unchanged after 10 min of trypsinization, indicating that the catalytic activity of phosphatase was not affected. At the end of 20 min, however, there was a slight decrease in phosphatase activity; apparently, prolonged trypsinization impaired the catalytic activity. Panel B shows a parallel experiment in which the enzyme was trypsinized in the presence of EGTA. Phosphatase activity increased with the time of trypsinization, and the enzyme activity assayed in Ca^{2+} was again higher than that

[1] Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; CaM, calmodulin; Tris, tris(hydroxymethyl)aminomethane.

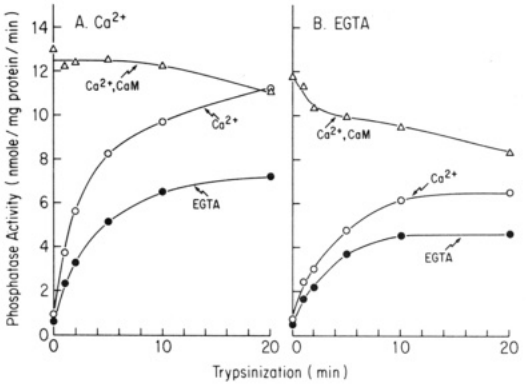

FIGURE 1: Stimulation of phosphatase by trypsinization in the presence or absence of Ca^{2+}. Phosphatase (8.8 μg) was incubated at 30 °C with trypsin (0.09 μg) in 50 mM Tris-HCl (pH 7.0)–0.5 mM DTT in the presence of 0.1 mM Ca^{2+} (panel A) or 1 mM EGTA (panel B). Trypsinization was terminated with soybean trypsin inhibitor (inhibitor:protease = 4:1 w/w) at the times indicated; aliquots were immediatly assayed for phosphatase in the presence of 1 mM EGTA (●), 0.1 mM Ca^{2+} (○), or 0.1 mM Ca^{2+} and 1.2 μM calmodulin (△). Phosphatase concentration in the assay mixture was 25.5 nM. In zero-time samples, trypsin inhibitor was added prior to trypsin. Other conditions were as described under Materials and Methods.

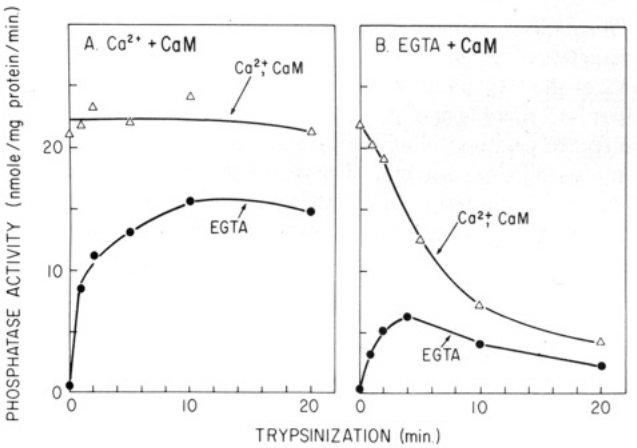

FIGURE 2: Trypsinization of phosphatase in the presence of calmodulin. Phosphatase (9.1 μg) and calmodulin (95 μg) were incubated at 30 °C with trypsin (0.9 μg) in 50 mM Tris-HCl (pH 7.0)–0.5 mM DTT in the presence of 0.1 mM Ca^{2+} (panel A) or 1 mM EGTA (panel B). At the times indicated, digestion was terminated with a 4-fold excess of trypsin inhibitor, and aliquots were immediately assayed for phosphatase activity in the presence of 1 mM EGTA (●) or 0.1 mM Ca^{2+} and 1.2 μM exogenous calmodulin (△). Phosphatase concentration in the assay mixture was 25.5 nM. In zero-time samples trypsin inhibitor was added prior to trypsin.

in EGTA. However, the highest levels of activity, when assayed in EGTA or in Ca^{2+}, were correspondingly lower than those trypsinized in the presence of Ca^{2+}, shown in panel A. In addition, phosphatase activity assayed with Ca^{2+} and calmodulin (upper curve) was also lower, indicating that in the absence of Ca^{2+} phosphatase is more susceptible to proteolysis. This finding is the more striking since trypsin is more active in the presence of Ca^{2+}. These results show that trypsinization of calmodulin-dependent phosphatase proceeded in two phases: a rapid activation by limited proteolysis and a slow inactivation upon prolonged proteolysis; Ca^{2+} seemed to slow down the process of inactivation.

To determine whether calmodulin had any effect on the trypsinization of phosphatase, the enzyme was treated with trypsin in the presence of calmodulin, either with or without Ca^{2+} (Figure 2). When trypsinized in the presence of Ca^{2+} (panel A), phosphatase activity assayed in EGTA increased with the time of trypsinization (lower curve); in contrast,

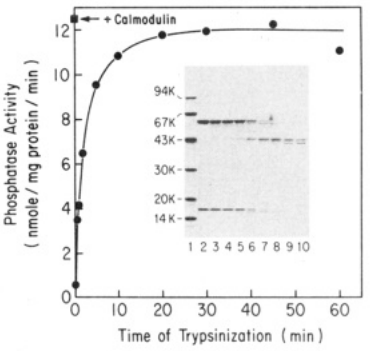

FIGURE 3: Time course of trypsinization of phosphatase. Phosphatase (31 μg) was incubated at 30 °C with trypsin (0.3 μg) in 50 mM Tris-HCl (pH 7.0), 0.5 mM DTT, and 0.1 mM Ca^{2+}. At the times indicated, aliquots were either added to a 4-fold excess of soybean trypsin inhibitor, to be assayed for phosphatase activity, or boiled in 1% SDS–5% β-mercaptoethanol, to be analyzed by gel electrophoresis. Phosphatase (23.3 nM) was assayed in the presence of Ca^{2+}, as described under Materials and Methods; the concentration of calmodulin, where indicated, was 1 μM. Inset: phosphatase (3 μg) after denaturation was analyzed on a 7.5–15% polyacrylamide gel, in the presence of 0.1% SDS and 1 mM EGTA, as described in the text. (Lane 1) Molecular weight markers: phosphorylase *b* (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), trypsin inhibitor (20 K), and α-lactalbumin (14.4K). (Lanes 2–10) Phosphatase trypsinized for 0, 0.5, 1, 2, 5, 10, 20, 40, and 60 min, respectively. The anode is at the bottom of the gel.

phosphatase activity assayed in Ca^{2+} and calmodulin (upper curve) remained essentially unchanged. These results are similar to those obtained with phosphatase trypsinized in the presence of Ca^{2+} alone (Figure 1A). When phosphatase was trypsinized and assayed in the presence of EGTA (panel B), there was only a slight increase in phosphatase activity (lower curve). When assayed in Ca^{2+} and calmodulin (upper curve), phosphatase activity decreased rapidly with the time of trypsinization; at the end of 20 min, only about 20% of the phosphatase activity remained. Since calmodulin and phosphatase form a complex in the presence of Ca^{2+} and not in its absence, these data suggest that phosphatase complexed with calmodulin is more resistant to inactivation by proteolysis.

Changes in Molecular Size as a Function of the Extent of Trypsinization. To follow the molecular change of phosphatase during the course of proteolysis, the enzyme was incubated with trypsin for various times, one aliquot was assayed for phosphatase activity, and another was analyzed by SDS–polyacrylamide gel electrophoresis. As shown in Figure 3, phosphatase activity increased with the time of trypsinization, reached maximum activity after 20 min, and remained elevated for an additional 40 min. The native enzyme in lane 2 (inset) displays the typical two subunits of M_r 60 000 (A) and 16 500 (B). Lanes 3–10 show the gel patterns of phosphatase after various times of tryspinization and the changes in the molecular weights of both subunits. At the early stages of trypsinization, subunit A appeared to have been converted to a protein of 59 000 daltons which was not clearly separated from subunit A (lanes 3–5) but was visible at later times (lanes 6–8), at which times a 43 000-dalton peptide was already apparent. After 20 min of trypsinization, phosphatase was fully activated, and the predominant proteolytic product of subunit A was the species of 43 000 daltons (lane 8). Upon futher trypsinization, this species was degraded to a 41 000-dalton protein (lanes 9 and 10) with no significant loss of enzyme activity. The time course of proteolysis of subunit B was different from that of subunit A. After 2 min of trypsinization, when phosphatase was activated some 50%, subunit B appeared intact while subunit A was partially degraded to the 43 000-dalton species (lane 5). After 5 min of trypsini-

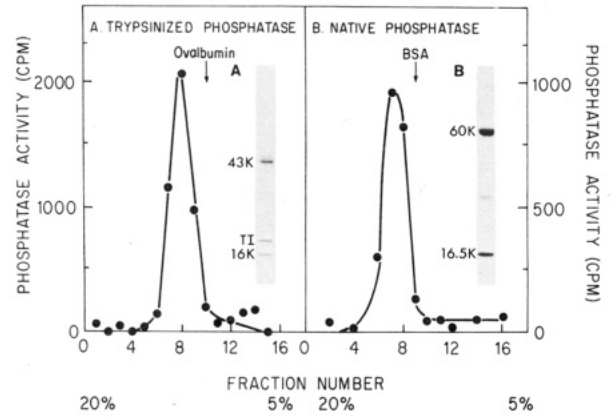

FIGURE 4: Sucrose density gradient centrifugation of trypsinized phosphatase. Phosphatase was trypsinized for 20 min as described under Materials and Methods. One-tenth milliliter of a solution containing 17.2 μg of trypsinized phosphatase and 2 μg of $^{14}CH_3$ methylated ovalbumin (panel A) or 6.9 μg of native phosphatase and 2 μg of $^{14}CH_3$ methylated bovine serum albumin (panel B) was layered onto a 5–20% sucrose density gradient in 50 mM Tris-HCl (pH 7.0), 0.5 mM DTT, and 0.1 mM Ca^{2+} and centrifuged at 45 000 rpm for 16 h at 4°C in a Beckman SW50.1 rotor. The tube was punctured at the bottom, and fractions of 250 μL were collected manually. Aliquots were analyzed for ^{14}C radioactivity or assayed for phosphatase in the presence of Ca^{2+} (trypsinized enzyme) or Ca^{2+} and 1.3 μM calmodulin (native enzyme). Insets: The peak activity fractions of trypsinized phosphatase (tube 8, panel A) and native phosphatase (tube 7, panel B) were analyzed on a 7.5–15% polyacrylamide gel in the presence of 0.1% SDS and 1 mM EGTA. The anode is at the bottom of the gel. TI, trypsin inhibitor.

zation (lane 6), subunit B partially disappeared, and a 16 000-dalton species appeared; at later times (lanes 7–10), both subunit B and the 16 000-dalton protein disappeared, giving rise to lower molecular weight peptides. Such a preparation, however, retained full phosphatase activity, implying that intact subunit B was not necessary for enzymic activity. This notion is in accord with other experiments demonstrating that the catalytic site of phosphatase resides in subunit A.[2] Limited proteolysis of phosphatase was examined previously on polyacrylamide gels by Klee & Krinks (1978), who noted similar changes in subunit A but detected no cleavage of subunit B. The discrepancy may be due to differences in the conditions of proteolysis or gel electrophoresis.

Since trypsinization degrades both phosphatase subunits to smaller moelcular weight peptides, we determined whether proteolysis dissociated the two subunits. Phosphatase was trypsinized for 20 min to give a fully activated enzyme and then sedimented on a 5–20% linear sucrose gradient (Martin & Ames, 1961). As shown in Figure 4A, phosphatase activity was recovered as a single symmetrical peak corresponding to a sedimentation coefficient of 4.26 S and a molecular weight of 60 000. In a parallel tube, the native enzyme gave a sedimentation coefficient of 4.65 S, corresponding to a molecular weight of 80 000 (Figure 4B), as was previously reported (Sharma et al., 1979; Wallace et al., 1979). Identical results were obtained whether the sucrose density gradient contained Ca^{2+} or EGTA. Analysis by SDS–polyacrylamide gel electrophoresis of the tube containing the peak activity of trypsinized phosphatase (inset) revealed two protein bands of 43 000 and 16 000 daltons, suggesting that limited trypsinization may not have dissociated phosphatase into its subunits. The possibility that the 16 000-dalton protein was a degradative fragment of subunit A cannot be excluded; however, this does not appear likely in view of the protein patterns shown in Figure 3.

[2] M. A. Winkler, D. Merat, E. A. Tallant, S. Hawkins, and W. Y. Cheung (unpublished results).

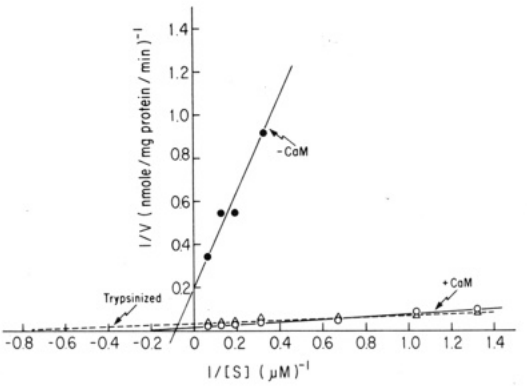

FIGURE 5: Effect of substrate concentration on trypsinized phosphatase activity. Phosphatase (25 nM) was assayed in a standard reaction mixture containing various amounts of ^{32}P-labeled casein. The substrate concentration is expressed on the basis of ^{32}P-labeled casein. Trypsinization and assay of phosphatase are described under Materials and Methods. Phosphatase in the absence of calmodulin (●); phosphatase in the presence of 1.3 μM calmodulin (○); trypsinized phosphatase (△).

Effect of Trypsinization on the Kinetic Parameters of Phosphatase. The effect of trypsinization on the kinetic parameters of the phosphatase was examined in Figure 5. The K_m of the trypsinized phosphatase was 1.2 μM, and the V_{max} was 30.9 nmol (mg of protein)$^{-1}$ min^{-1}. As a comparison, the effect of calmodulin on the kinetic parameters was included in a parallel experiment. The K_m of the native enzymes for casein in the absence of calmodulin was 10.8 μM, and in its presence, it was 4.2 μM; the corresponding V_{max} values were 4.9 and 71.6 nmol (mg of protein)$^{-1}$ min^{-1}, respectively. The basal phosphatase activity (in the absence of calmodulin) was uniformly low, and it is especially difficult to obtain accurate measurements with low substrate concentrations. Thus, the kinetic parameters calculated from this curve are, at best, estimates. Although trypsinization significantly increased the V_{max}, it was less than that of the calmodulin-stimulated activity. Qualitatively similar results were obtained with phosphodiesterase (Cheung, 1971) and myosin light chain kinase (Srivastava & Hartshorne, 1983; Walsh et al., 1982).

Loss of Calmodulin Sensitivity of Trypsinized Phosphatase. Calmodulin stimulated phosphatase activity by forming a Ca^{2+}-dependent complex with the enzyme (Tallant & Cheung, 1983a). This property has been effectively exploited to purify the enzyme on a calmodulin–Sepharose affinity column. To determine whether the trypsinized phosphatase still retains the ability to bind to calmodulin, proteolyzed phosphatase was chromatographed on a calmodulin–Sepharose column (Figure 6). In the control experiment (panel A), the native enzyme bound to the column in the presence of Ca^{2+} and was eluted with EGTA; the eluted enzyme was sensitive to calmodulin. After treatment with trypsin for 20 min, the majority of phosphatase did not bind to the column and appeared in the flow-through fractions (panel B); a small fraction of enzyme activity bound to the column and was eluted with EGTA, and this phosphatase activity was slightly stimulated by calmodulin, implying that the enzyme may have been incompletely trypsinized. After a 60-min incubation with trypsin, essentially all phosphatase activity appeared in the flow-through fractions (panel C). The trace of phosphatase activity eluted by EGTA was not sensitive to calmodulin. When analyzed by SDS–polyacrylamide gel electrophoresis, the unbound phosphatase from panel B showed protein bands of 43 000 and 16 000 daltons, similar to the results of sucrose density gradient

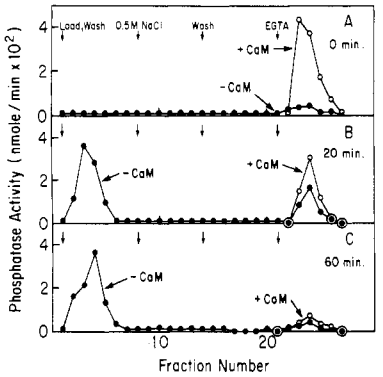

FIGURE 6: Calmodulin–Sepharose chromatography of trypsinized phosphatase. Calmodulin–Sepharose was prepared by covalently coupling bovine brain calmodulin to Sepharose 4B that had been activated by cyanogen bromide (Klee & Krinks, 1978). The extent of coupling was 3–4 mg of calmodulin per mL of resin. The column (0.25 × 2 cm) was equilibrated in buffer A [50 mM Tris-HCl (pH 7.8), 3 mM $MgSO_4$, 0.5 mM DTT, and 0.02% NaN_3] containing 0.1 mM $CaCl_2$. Phosphatase was either not treated with trypsin (panel A) or degraded with trypsin for 20 (panel B) or 60 min (panel C). One hundred microliters of sample in buffer A was loaded onto the column in the presence of 0.1 mM Ca^{2+} and was subsequently eluted with 2-mL aliquots of buffer A containing (1) 0.1 mM Ca^{2+}, (2) 0.1 mM Ca^{2+} and 0.5 M NaCl, (3) 0.1 mM Ca^{2+}, and (4) 1 mM EGTA. Fractions of 0.3 mL were collected. Phosphatase was assayed as described under Materials and Methods in the presence (○) or absence (●) of 1.3 μM calmodulin. Recovery of phosphatase activity following chromatography was 66% (A), 78% (B), and 81% (C) of the applied activity.

Table I: Effect of Ca^{2+} and Mn^{2+} on Phosphatase Activity[a]

other additions	activity [nmol (mg of protein)$^{-1}$ min^{-1}] native phosphatase	trypsinized phosphatase
none	0.5	7.5
calmodulin	9.4	8.3
EGTA (1 mM)	0.7	4.6
EGTA + calmodulin	0.8	4.1
$MnCl_2$ (0.5 mM)	1.2	17.7
$MnCl_2$ + calmodulin	17.2	17.5

[a] The reaction mixture contained the usual ingredients and other additions, as indicated above. The concentration of calmodulin was 1.1 μM, and that of phosphatase was 13.8 nM. Phosphatase was trypsinized and assayed as described under Materials and Methods.

centrifugation in Figure 4. Klee & Krinks (1978) noted that limited proteolysis of the phosphatase resulted in complete loss of its ability to inhibit calmodulin-dependent phosphodiesterase activity.

Comparison between the Effect of Ca^{2+} and Mn^{2+} on Phosphatase Activity. The effects of Ca^{2+} and Mn^{2+} on the activities of the native and trypsinized phosphatase were compared in Table I. After full activation by trypsin, the proteolyzed phosphatase was relatively insensitive to calmodulin; on the other hand, the native enzyme was stimulated some 19-fold by calmodulin. The trypsinized phosphatase was, however, still sensitive to Ca^{2+} as its activity was reduced 40% in the presence of EGTA. Mn^{2+} further stimulated the proteolyzed enzyme activity 2-fold; this stimulation was independent of calmodulin. Mn^{2+} also proved to be more effective than Ca^{2+} in stimulating the native enzyme, increasing both the basal and calmodulin-stimulated activites from 1- to 2-fold.

Using a Ca^{2+}–EGTA buffer, we examined the Ca^{2+} requirement of the trypsinized phosphatase. Figure 7 shows the titration curve of the Ca^{2+}-supported trypsinized phosphatase activity; the curve was hyperbolic, and half-maximal stimulation was obtained at 0.34 μM Ca^{2+}. A Hill plot of these data (not shown) revealed a Hill coefficient of 1.0, demonstrating a lack of cooperativity. As a comparison, the titration curve of the native phosphatase was included. Although half-maximal stimulation was obtained similarly at 0.35 μM Ca^{2+}, the curve was sigmoidal, and the Hill coefficient was 4.9 (data not shown), indicating strong positive cooperativity.

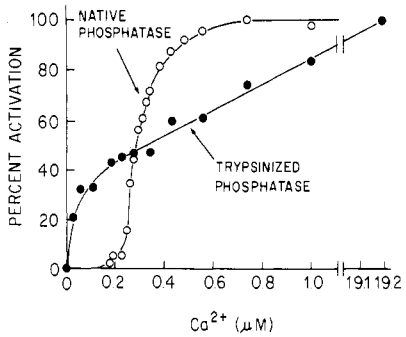

FIGURE 7: Activation of trypsinized phosphatase by Ca^{2+}. Phosphatase was assayed in a standard reaction mixture containing 2 mM EGTA, 26 nM phosphatase, and various amounts of $CaCl_2$ to obtain the indicated level of free Ca^{2+}. Phosphatase was trypsinized and then dialyzed against 50 mM Tris-HCl (pH 7.0)–0.5 mM DTT containing 2 mM EGTA. Trypsinization of phosphatase, the phosphatase assay, and the Ca^{2+}–EGTA buffer are as described under Materials and Methods. Phosphatase activity is expressed as percent activation. Native phosphatase was assayed in the presence of 290 nM calmodulin (○); basal activity, 0.4 nmol (mg of protein)$^{-1}$ min^{-1}; fully stimulated enzyme activity, 14.0 nmol (mg of protein)$^{-1}$ min^{-1}. The stimulated activity, 13.6 nmol (mg of protein)$^{-1}$ min^{-1}, was taken as 100% activation. Trypsinized phosphatase activity (●) in 1 mM EGTA was 11.2 nmol (mg of protein)$^{-1}$ min^{-1} and in 0.1 mM Ca^{2+} 15.1 nmol (mg of protein)$^{-1}$ min^{-1}. The difference, 3.9 nmol (mg of protein)$^{-1}$ min^{-1}, represents the Ca^{2+}-stimulated trypsinized phosphatase activity and is normalized to 100% activation as given in the figure.

Discussion

We have shown that calmodulin-dependent protein phosphatase isolated from bovine brain is stimulated by limited trypsinization, in a manner analogous to other calmodulin-dependent enzymes (Cheung, 1971, 1981; Depaoli-Roach et al., 1979; Carafoli et al., 1982; Walsh et al., 1982; Srivastava & Hartshorne, 1983). Extensive trypsinization, however, causes inactivation of phosphatase. Ca^{2+} slows down the process of tryptic inactivation, suggesting that subunit B, which is a Ca^{2+}-binding protein, protects the catalytic site. Calmodulin further protects the enzyme from proteolytic degradation; interaction between the apoenzyme and the Ca^{2+}–calmodulin complex probably causes the enzyme to assume a new conformation such that the catalytic site is more protected from the protease.

Calcium-dependent complex formation between calmodulin and phosphatase has previously been correlated with an increase in thermal stability (Wallace et al., 1979). In that study, calmodulin binding to phosphase was monitored by inhibition of calmodulin-dependent phosphodiesterase activity. In the presence of Ca^{2+} and calmodulin, the phosphodiesterase inhibitory activity was more resistant to thermal inactivation, suggesting a conformational change as a result of protein–protein interaction. Similar results have been observed with phosphodiesterase (Liu & Cheung, 1976) and with rat brain adenylate cyclase (Lynch et al., 1977).

Trypsinization reduces the molecular weight of phosphatase, with both of its subunits undergoing some proteolytic cleavage; a parallel change is the loss of sensitivity to calmodulin. These data suggest that limited proteolysis of the phosphatase releases fragments from the enzyme, causing it to assume a new con-

formation with the catalytic site more exposed to the substrate. The released fragments may contain the calmodulin-binding domain, accounting for the insensitivity of the enzyme to calmodulin. On the other hand, proteolysis may only alter the conformation such that calmodulin can no longer bind to phosphatase (Lin & Cheung, 1980).

Stimulation of phosphatase activity by calmodulin and by limited proteolysis produces an activated enzyme with comparable activities. The two agents also have qualitatively similar effects on the kinetic parameters of the phosphatase. These results do not necessarily mean that the two activated enzymes possess a similar conformation; rather, they suggest that limited tryptic cleavage of phosphatase allows the enzyme to reach an activity level comparable to that by a calmodulin-induced conformational change.

Although the trypsinized phosphatase is insensitive to calmodulin, it retained some sensitivity to Ca^{2+}. This could be due to subunit B, a Ca^{2+}-binding protein similar to but not identical with calmodulin (Aiken et al., 1982). In fact, the sensitivities of the Ca^{2+}-dependent activity of both the native and trypsinized phosphatase are comparable. Since the trypsinized phosphatase appears to retain subunit B, the similar Ca^{2+} sensitivity may reflect the intrinsic affinity of subunit B for Ca^{2+}. Similarly, phosphorylase kinase after trypsinization is insensitive to calmodulin but is partially sensitive to Ca^{2+} (Depaoli-Roach et al., 1979). Sensitivity to Ca^{2+} is probably conferred by its δ subunit, an intrinsic molecule of calmodulin.

The trypsinized phosphatase was further stimulated by Mn^{2+}, as was basal and calmodulin-stimulated phosphatase activity. Although Mn^{2+} can substitute for Ca^{2+} in calmodulin stimulation (Lin et al., 1974; Teo & Wang, 1973), the effect of Mn^{2+} on phosphatase appears to be independent of calmodulin and may be due to the metal binding sites on subunit B, or subunit A, or to facilitated interaction of phosphatase with its substrate.

The physiological relevance of the proteolytic activation of calmodulin-dependent phosphatase is unknown. An in vivo stimulation by endogenous proteases would result in irreversible activation of phosphatase and its loss of sensitivity to calmodulin, giving rise to calmodulin-independent activity. It is conceivable that phosphatase in vivo is totally dependent upon calmodulin for activity. Myosin light chain kinase from both smooth muscle and nonmuscle cells appears to be such an example (Dabrowska & Hartshorne, 1978; Waisman et al., 1978; Yagi et al., 1978). It will be of interest to determine whether phosphatase can be isolated totally dependent on calmodulin for activity.

After the manuscript had been completed, Manalan & Klee (1983) reported the activation of calmodulin-dependent protein phosphatase by limited proteolysis. Although the results from the two laboratories are in general agreement, our results extend their findings. Moreover, some differences are apparent, and they may be due to slight differences in experimental procedures or assay conditions, or both.

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Registry No. Ca, 7440-70-2; Mn, 7439-96-5; phosphoprotein phosphatase, 9025-75-6; trypsin, 9002-07-7.

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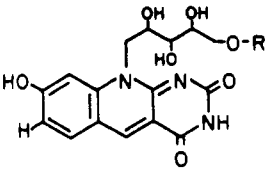
Properties of 7,8-Didemethyl-8-hydroxy-5-deazaflavins Relevant to Redox Coenzyme Function in Methanogen Metabolism†

Fredric Jacobson‡ and Christopher Walsh*

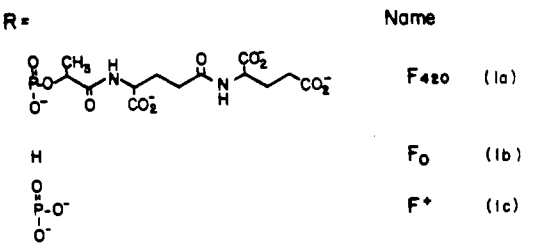
ABSTRACT: The 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) moiety is the key element in the newly discovered redox coenzyme factor 420 (F420) found in methanogenic bacteria and in streptomycetes. In this paper, we have analyzed chemical properties of synthetic FO that condition coenzyme function and compare FO to 5-deazariboflavin (5-dRF) and 8-hydroxyriboflavin. The equilibrium constants for sulfite addition and the rates of reoxidation of FOH_2 by a series of flavin analogues show that the 5-carba substitution imposes nicotinamide-like chemistry on the system, including sluggishness to reoxidation by O_2. Ionization of the 8-OH substituent in the oxidized FO (pK_a = 5.85) suppresses reactivity of FO toward redox chemistry. In the reduced FOH_2, the phenolic group is isolated and shows a more normal pK_a of 9.7. The reduction potential of FO/FOH_2 has been determined by equilibration with two methanogen enzymes, an F420-reducing hydrogenase and an NADP:F420 oxidoreductase, to be −340 to −350 mV. The rate of the bimolecular disproportionation of FOH_2 and FO was followed by high-pressure liquid chromatography analysis, starting with tritium in the oxidized species, and shown to be 10–20 M^{-1} min^{-1}, down 50–100-fold from the 5-dRF/5-$dRFH_2$ reaction. This extended lifetime of chiral [5-^{3}H]FOH_2 samples in the presence of FO molecules permits stereochemical determination of hydride transfers to and from C(5) of the 8-hydroxy-5-deazaflavin system. Methanogen hydrogenase and NADP:F420 oxidoreductase are defined to show "A" side specificity while the NAD:FMN oxidoreductase from *Benekea harveyii* shows "B" side specificity.

In addition to the characteristics that distinguish all Archaebacteria from other Procaryotae (Eubacteria) (Balch et al., 1977, 1979; Woese et al., 1978), the methane-producing bacteria possess a variety of apparently unique cofactors involved in electron and one-carbon transport. These include the nickel tetrapyrrole F430 (Gunsalus & Wolfe, 1978; Whitman & Wolfe, 1980; Diekert et al., 1980a–c), coenzyme M (2-mercaptoethanesulfonate) (Taylor & Wolfe, 1974), F342 (Gunsalus & Wolfe, 1978), and the yellow fluorescent cofactor (YFC) (Daniels, 1978). Methanogens also contain the redox-active cofactor F420[1] (I) (Cheeseman et al., 1972; Eirich et al., 1979), which has been identified as a derivative of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) (II) (Eirich et al., 1978; Ashton et al., 1979). This molecule is used to link H_2 or formate oxidation to reduction of NADP (Tzeng et al., 1975a,b) and perhaps to serve as reductant in the last step of methane biosynthesis (Ellefson & Wolfe, 1980, 1981).

This natural product 5-deazaflavin is not restricted to the methanogens. Eker and his co-workers have recently found the chromophore in cell extracts of *Streptomyces griseus* and in a homogeneous preparation of its thymine dimer cleaving photoreactivation enzyme (PRE) (Eker et al., 1980, 1981; Eker, 1980). As long ago as 1960, McCormick and co-workers described a "cosynthase" factor, with the same spectroscopic features as F420, involved in chlortetracycline biosynthesis by another *Streptomyces* strain (McCormick et al., 1960; Miller et al., 1960) and have now assigned to it an 8-hydroxy-5-deazaflavin structure (McCormick & Morton, 1982). Prelim-



7,8-didemethyl-8-hydroxy-5-deazariboflavin (oxidized)



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[1] Abbreviations: FO, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; F^+, 5′-phosphate of FO; F420, the natural product methanogen deazaflavin cofactor; RF, riboflavin; 5-dRF, 5-deazariboflavin; 8-OH-5-dRF, 8-demethyl-8-hydroxy-5-deazariboflavin; 8-OH-5-dFl_{Et}, *N*(10)-ethyl-8-hydroxy-5-deazaisoalloxazine; 8-MeO-5-dFl_{Et}, *N*(10)-ethyl-8-methoxy-5-deazaisoalloxazine; H_2ase, hydrogenase; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; MES, 4-morpholineethanesulfonic acid; HPLC, high-pressure liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid. In FOH_2 and 5-$dRFH_2$, the H_2 suffix denotes that the flavin is in the 1,5-dihydro form.

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