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PURIFICATION OF A MEMBRANE-DERIVED PROTEINASE CAPABLE OF ACTIVATING A GALACTOSYLTRANSFERASE INVOLVED IN VOLUME REGULATION

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UDPgalactose:*sn*-glycerol-3-phosphate α-D-galactosyltransferase (IFP-synthase, EC 2.4.1.96) shows low activity in extracts prepared from standard volume cells of *Poterioochromonas malhamensis* under certain conditions. This inactive enzyme has been partially purified by chromatography on DEAE-cellulose, Sephadex G-150 and α-lactalbumin-agarose. It can be activated by an auxiliary enzyme which can be eluted from membranes and which has been purified to homogeneity by chromatography on DEAE-Sephacel and immobilized hemoglobin and fetuin. The activating enzyme is inhibited by chymostatin, antipain and diisopropylfluorophosphate and does not require divalent ions. It consists of a single peptide chain of molecular weight 46 000, can split certain proteins and appears to be a serine proteinase operating around a pH of 6.0. The activating proteinase is irreversibly generated in the crude homogenates on addition of Ca^{2+} and also shows increased activity shortly after cell shrinkage. This might indicate that it represents one of the possibilities to render the galactosyltransferase active as a result of the physiological stimulus.

Introduction

Addition of solutes to a suspension of the golden-brown wall-less alga *Poterioochromonas malhamensis* causes the cells to shrink within about 1 min. An increase from 65 to 180 mosmol/kg, for instance, reduces the volume by about 20%. The lost water is regained within 1–2 h due to an internal accumulation of solutes, mainly of isofloridoside (α-galactosyl 1-glycerol) [1]. This substance is synthesized by dephosphorylation of isofloridoside phosphate (α-galactosyl 1-glycerol 3 phosphoric acid) which in turn result from action of the soluble enzyme IFP-synthase. One form of this enzyme has recently been purified to homogeneity [2]. It remains, however, questionable whether this active enzyme, as such, is present in live cells of standard volume. To overcome the instability of the enzyme, the cells were homogenized in the presence of 0.1% (w/v) cetyltrimethylammonium bromide and 30% (w/v) glycerol, which seriously diminished cell volume prior to homogenization. It has been previously shown by chase experiments with [^{14}C]glucose that the formation of isofloridoside is low in cells of standard volume and rapidly increases after cell shrinkage [3]. This has been taken as an indication that the IFP-synthase might be a major site of regulation of the isofloridoside pathway. Extracts of standard volume cells were also found to exhibit low IFP-synthase activity, whereas the activity of this enzyme was higher as cell shrinkage increased [4,5].

On the basis of such observations, we have proposed that cell shrinkage might trigger activa-

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Abbreviations: IFP-synthase, UDPgalactose : *sn*-glycerol-3-phosphate α-D-galactosyltransferase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholinoethanesulfonic acid.

tion of IFP-synthase [1,4–6]. In order to characterize a relevant mechanism, we have tried to optimize the conditions of cell desintegration in order to reflect the physiological situation, namely to obtain homogenates with low IFP-synthase activity from cells of standard volume. This was achieved when we homogenized the cells in a Yeda-press in the presence of 2 mM EDTA, bovine serum albumin, 3.3 mM 2-mercaptoethanol, high buffer concentration and at a pH value of 6.2 [4,5]. In such extracts the inactive IFP-synthase could be rendered active by proteolysis with trypsin or chymotrypsin [7]. We also could demonstrate activation of the IFP-synthase in such crude homogenates by an endogenous auxiliary enzyme. Generation of this enzyme required the presence of membranes and could be started irreversibly by addition of Ca^{2+}; this effect was increased when calmodulin, isolated from *Poterioochromonas* or bovine brain, was added [5,6]. Alternatively, low concentrations of certain detergents or detergent-like phenothiazine drugs also led to the appearance of the activating enzyme [5,8].

This report concerns itself with the purification to homogeneity of a membrane-derived enzyme capable of activating the inactive IFP-synthase. It appears to be a specific proteinase which might represent one possibility to render the IFP-synthase active as a consequence of osmotically induced cell shrinkage.

Materials and Methods

Materials. Most of the materials were as described before [2,6]. Hemoglobin according to Anson (Merck, Darmstadt) was coupled to Sepharose CL-6B (Pharmacia) with 1,1′-carbonyldiimidazol (Fluka) [9]. Fetuin-agarose (type III-A), α-lactalbumin-agarose, fetuin type III, oxidized bovine insulin A- and B-chain as well as the inhibitors and other proteins listed in Table III were bought from Sigma. The fluorogenic and chromogenic proteinase substrates were purchased from Sigma, Bachem or Merck (Darmstadt). Fluorescamine ('Fluram') was obtained through Hoffmann-La Roche (Basel).

Enzyme assays. IFP-synthase was assayed with 25 μl of 100 mM Hepes-NaOH, pH 7.8, in a total volume of 50 μl by measuring the amount of galactosyl groups transferred from UDPgalactose to *sn*-[^{14}C]glycerol 3-phosphate, as described previously [2,6]. After addition of a 10 μl aliquot of enzyme exhibiting a pH value of 6.0 to 6.2, a final pH of 7.5 to 7.6 was achieved in the assay mixture. These pH values were directly measured with a Radiometer microelectrode type E 5021. Assays were run at 25°C for 1–5 min.

1 unit of IFP-synthase is the amount of enzyme that produces 1 μmol of [^{14}C]isofloridoside phosphate per minute.

For assay of the IFP-synthase activating enzyme the IFP-synthase was adjusted to pH 6.0 through the addition of 0.1 M maleic acid and mixed with 1 volume of activating enzyme in 30 mM Mes-NaOH, pH 6.0. To determine the pH optimum of the activating enzyme various amounts of either 0.1 M maleic acid or NaOH were added and the actual values were measured as above. The mixture was incubated at 25°C and 10 μl aliquots were removed at various times and immediately added to the IFP-synthase assay mixture, as described above. Routinely, various dilutions of the activating enzyme were run in parallel to find the concentration where the increase in IFP-synthase activity was roughly proportional to time during 10–15 min (see Fig. 1). For inhibition studies the purified activating enzyme was preincubated with the inhibitors for 5–10 min at 25°C.

1 unit of activating enzyme is defined as that activity which increased the IFP-synthase activity by 1 unit per min.

When the activating enzyme had to be determined in crude homogenates only 5 g/l bovine serum albumin were added to the homogenization buffer (see below). Aliquots of homogenate or supernatant were mixed at 0°C with the 10-fold concentrated inhibitor (or water for the control), immediately incubated at 25°C and samples for IFP-synthase determination taken in 1 min intervals. The activity given was calculated from the increase in IFP-synthase over 3 min. It reflects the activity present in the cells before homogenization as well as some enzyme liberated spontaneously during the incubation at 25°; this tendency is more pronounced for membranes from shrunken cells [5].

Cell homogenization. Poterioochromonas malhamensis Peterfi (syn. *Ochromonas malhamensis*

Pringsheim) was grown in the light and cell suspensions exhibiting 65 mosmol/kg were prepared as described before [6]. The suspension was aerated at 27°C for about 30 min and the cells sedimented at 2000 × *g* for 10 min. The pellet was suspended in ice-cold buffer (100 mM Mes-NaOH, pH 6.2, 2 mM EDTA, 3.3 mM 2-mercaptoethanol, 15 g/l bovine serum albumin), quickly homogenized at 100 bar in a cooled Yeda-press and centrifuged at 48 000 × *g* for 10 min and 4°C. All further steps were performed at 4°C.

Purification of inactive IFP-synthase. Cells from 400 ml of suspension were resuspended in 200 ml of homogenization buffer and homogenized as described above. The supernatant was applied to a DEAE-cellulose column (2.5 × 35 cm) equilibrated with 30 mM Tris-HCl, pH 8.0, 14 mM 2-mercaptoethanol, 0.05% (w/v) NaN_3. The column was first washed at 325 ml/h with the buffer and then eluted with 600 ml of a 0–0.5 M NaCl gradient in the same buffer. Fractions with activity were pooled and concentrated to about 12 ml in an Amicon ultrafiltration cell and chromatographed in the above buffer with 30 ml/h on a Sephadex G-150 column (5 × 100 cm), collecting fractions of 6 ml. The fractions with IFP-synthase activity were again pooled, concentrated as above to about one third and stored in 3–5 ml portions in liquid nitrogen. These portions were thawed if required and applied on a 10 ml α-lactalbumin-agarose column (1 cm diameter). The column was washed with the above mentioned Tris-buffer and eluted with a 0–0.5 M NaCl gradient (15 ml/h, 30 ml). The fractions exhibiting IFP-synthase activity were pooled and concentrated, if necessary.

Purification of IFP-synthase activating enzyme. The cells (290–600 ml) were homogenized in an equal volume of buffer, centrifuged as described above, and the walls of the centrifuge tubes cleaned. The pellet was resuspended on a Vortex in ice-cold distilled water (one fifth of the suspension volume). The activating enzyme was found in the supernatant of a further centrifugation step (48 000 × *g*, 10 min). It was applied on a DEAE-Sephacel column (2.2 × 17.5 cm) equilibrated with 30 mM Mes-NaOH, pH 6.0. After washing with the same buffer, the column was eluted with a 0–0.5 M NaCl gradient (100 ml, 50 ml/h) and the active fractions pooled and dialysed against the above buffer. An aliquot (15 ml) of the solution was applied on a hemoglobin-Sepharose column (1 × 5 cm), washed, and eluted with a gradient from 30 mM Mes-NaOH, pH 6.0, to 30 mM Tris-HCl, pH 8.1 (50 ml, 20 ml/h). The fractions containing activating enzyme were pooled, dialysed against the above buffer and stored at −20°C.

For affinity chromatography on fetuin-agarose either one third of the dialysed DEAE-Sephacel pool was used directly, or the total pooled active fractions from the hemoglobin-Sepharose step were used and applied on a 10 ml column (1 cm diameter) equilibrated with 30 mM Mes-NaOH, pH 6.0. After washing with the same buffer, elution was performed with a 0–1.5 M NaCl gradient (40 ml, 65 ml/h). The fractions with activating enzyme were dialysed against the above buffer, concentrated and stored at −20°C.

Determination of molecular weight. This was performed either on SDS-polyacrylamide gel electrophoresis with 10% acrylamide gel plates, 0.5 cm thick [10] or on a Sephadex G-150 column (1.6 × 92 cm) equilibrated with 30 mM Mes-NaOH, pH 6.0.

Determination of primary amino groups. The sample (50 µl) was diluted with 950 µl of 50 mM sodium borate buffer, pH 9.0, and then 500 µl of fluorescamine (15 mg/100 ml acetone) were quickly mixed in [11]. Relative fluorescence was measured in a Jobin-Yvon JY 3D spectrofluorometer (excitation 398 nm, emission 508 nm). A calibration curve was established using glycine as a standard (0–4 nmol).

Proteinase assays. The nitroanilides (0.8 ml, 1 mM or saturated, if not soluble, in 30 mM Mes-NaOH, pH 6.0) were incubated with 100 µl (5 µg) of the activating enzyme for 2 h at 25°C and A_{410} determined. Alternatively amidomethylcoumarins were used and the relative fluorescence determined (excitation 380 nm, emission 460 nm). When naphthylamides were used as substrates, liberation of amino groups was followed with the above fluorescamine assay.

Protein determination. Protein was determined according to Sedmak and Grossberg [12] using bovine serum albumin as a standard.

Results

Purification of inactive IFP-synthase

To have a storable substrate available for assay of the activating enzyme it was necessary to partially purify the IFP-synthase in its inactive form. This was achieved (Table I) by a combination of classical steps and chromatography on immobilized α-lactalbumin, a method originally introduced to purify the A-protein of lactose synthase [13]. Our result indicating that IFP-synthase also shows affinity towards this so-called 'specifier' protein might mean that α-lactalbumin has a more general property to bind to galactosyltransferases requiring UDPgalactose as a substrate. During purification of IFP-synthase we assayed the enzyme not only with the standard assay but also in the presence of 50 mM sodium pyrophosphate [4]. Under these conditions the activity of the enzyme was found to be 10 to 16 times higher than normal (Table I). When an aliquot of the enzyme purified on the α-lactalbumin-Sepharose (Table I) was supplied with 50 mM pyrophosphate and then dialyzed for 2 h, this activation was found to be fully reversible (data not shown). We have also observed that 100 mM EDTA can partially replace pyrophosphate; both chelators are almost inactive at 10 mM concentrations (data not shown). This suggests that the effect may be related to the chelating ability of both compounds. The extremely high concentrations which are necessary, however, indicate that not an inhibitory divalent ion which is freely exchangeable has to be removed. Although not understood, the effect of the pyrophosphate ions was of great help during the purification of the inactive IFP-synthase.

The inactive IFP-synthase could be stored after the Sephadex G-150 step at the temperature of liquid nitrogen. A loss of only 30% was observed after thawing, and this was independent of the time of storage up to half a year. Freshly thawed samples of this IFP-synthase preparation were used for the assays during purification of the activating enzyme and as a source for further purification on the α-lactalbumin column. After this step the IFP-synthase appeared to be more unstable and was used without further storage for the determination of the properties reported for the activating enzyme.

Purification of the activating enzyme

The purification procedure for the activating enzyme is based upon the previous experiments in which it showed low activity in homogenates prepared with high buffer concentrations and in the presence of EDTA and bovine serum albumin [4,5]. Additional experiments showed that it was active, and at least partly soluble, when homogenization buffer of low concentration was used (data not shown). We therefore simply eluted the enzyme by resuspending the crude membrane pellet in water. This allowed us to remove the soluble cellular proteins and the bovine serum albumin which had to be added in the homogenization buffer. Water was chosen as an eluent, as this left most of the membrane proteins insoluble and resulted in extracts whose specific activity was significantly higher when compared to extracts

TABLE I

PARTIAL PURIFICATION OF INACTIVE IFP-SYNTHASE

The enzyme was assayed during purification in the absence or presence of 50 mM sodium pyrophosphate ($\pm PP_i$). For a discussion of the PP_i effect and the degree of enzymatic activation of the inactive IFP-synthase (= − PP) see text or Fig. 1, respectively.

Fraction	Volume (ml)	Protein (mg)	IFP-synthase activity (units)		Spec. activity (U/g)		Recovery (%)		Purification (-fold)	
			$+PP_i$	$-PP_i$	$+PP_i$	$-PP_i$	$+PP_i$	$-PP_i$	$+PP_i$	$-PP_i$
Supernatant	204	3876	44.7	3.9	11	1	100	100	1	1
DEAE-cellulose	135	2025	28.2	1.8	14	0.9	67	46	1.3	0.9
Sephadex G-150	202	121	20.3	1.5	167	12.4	45	38	15	12.4
α-Lactalbumin-Sepharose [a]	196	8.6	10.6	1.0	1232	116	24	27	112	116

[a] Purification was performed with a 3 ml aliquot of the preceding step and the values given were calculated for the total fraction.

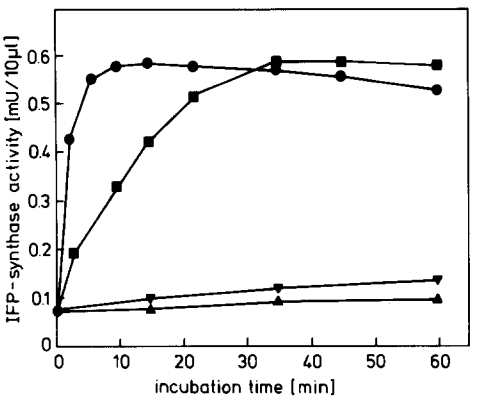

Fig. 1. Influence of time, concentration and temperature on the IFP-synthase activating enzyme. The IFP-synthase preparation from the α-lactalbumin-Sepharose step of Table I was mixed with an equal volume of purified activating enzyme (similar to the last step of Table II) to give 0 µg/ml (control, ▲), 0.5 µg/ml (■, ▼) or 1 µg/ml (●), final concentration. The mixtures were incubated at 25°C (●, ■, ▲) or 0°C (▼). Aliquots of 10 µl were withdrawn at the indicated times and assayed for IFP-synthase.

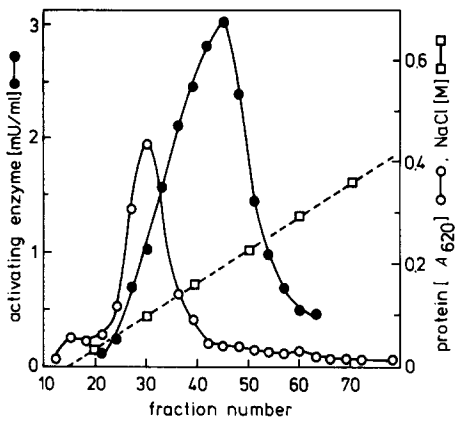

Fig. 2. Purification of the activating enzyme by chromatography on DEAE-Sephacel. The water extract from a crude membrane pellet corresponding to 700 ml homogenate was applied and treated as described in Materials and Methods. Fractions 36 to 52 were pooled. The values given for protein refer to a 50 µl aliquot out of the fractions.

made with Ca^{2+}-containing buffers or detergents (data not shown).

The principle for assay of the activating enzyme is illustrated by Fig. 1 which also provides evidence for the dependence of the activating reaction on time, temperature and enzyme concentration. The overall result of purification is summarized in Table II and the purification on an ion-exchange column is shown in Fig. 2. The chromatography on immobilized hemoglobin (Fig. 3) is based on a method described for the purification of cathepsin D [14] whereas fetuin was introduced as a ligand for affinity chromatography (Fig. 4) when we observed that this protein is a strong inhibitor of the activating enzyme (Table III). It should be pointed out that use of the two final chromatography steps was important. If the fetuin-agarose step was used alone, a preparation resulted exhibiting one major and 3–4 additional faint bands on SDS-gel electrophoresis. In contrast, only the major band and no impurities were found when the steps were combined in the way indicated in Table II.

Properties of the activating enzyme

The IFP-synthase activating enzyme was found to have a sharp optimum at pH-values between 5.9 and 6.1, with only 10 or 50% of the maximal activity attained at pH 5.5 or 6.5, respectively. It gave a single band with a molecular weight of

TABLE II

PURIFICATION OF THE IFP-SYNTHASE ACTIVATING ENZYME

The water extract from a crude membrane pellet corresponding to 290 ml of cell homogenate was fractionated as described in Material and Methods. Fractions were pooled as indicated in Figs. 2–4.

Fraction	Volume (ml)	Protein (mg)	Activating enzyme (units)	Spec. activity (units/mg)	Recovery (%)	Purification (-fold)
Membrane extract	50	95	1.59	0.017	100	1
DEAE-Sephacel	56	7.3	0.45	0.062	28	3.6
Hemoglobin-Sepharose	7.7	0.15	0.29	1.93	18	114
Fetuin-agarose	17.3	0.1	0.26	2.6	16	153

TABLE III

INHIBITION OF THE PURIFIED ACTIVATING ENZYME

An enzyme similar to the one mentioned in last line of Table II was used with 10 min of activation time. No inhibition was found with: sodium fluoride (100 mM), ammonium molybdate (0.1 mM), iodoacetamide (1 mM), *N*-ethylmaleimide (0.1 mM), EDTA (1 mM), EGTA (1 mM), pepstatin (0.4 mg/ml) leupeptin (0.4 mg/ml), bestatin (0.4 mg/ml), lima bean trypsin inhibitor (1 mg/ml), egg white trypsin inhibitor (0.4 mg/ml), bovine serum albumin (4 mg/ml), hemoglobin (4 mg/ml) cytochrome *c* (1 mg/ml), α-lactalbumin (1 mg/ml), lysozyme (1 mg/ml), bradykinine (1 mg/ml), bovine glucagon (1 mg/ml). The data given are the means from three independent experiments.

Substance added (final conc.)	Inhibition (%)
Diisopropylfluorophosphate (2 mM) [a]	62
Diisopropylfluorophosphate (5 mM) [a]	100
Phenylmethylsulfonylfluoride (2 mM) [b]	70
Chymostatin (2.5 μg/ml)	50
Antipain (37 μg/ml)	50
Fetuin (100 μg/ml)	54
Fetuin (1 mg/ml)	100
Casein (1 mg/ml)	67
Dimethylcasein (1 mg/ml)	73
Insulin, bovine, A-chain (1 mg/ml)	19
Insulin, bovine, B-chain (1 mg/ml)	89

[a] This substance also inhibited the IFP-synthase to some extent, therefore, inhibition was calculated from a control containing the substance but no activating enzyme.

[b] 30 min preincubation.

46 000 on SDS-polyacrylamide gel electrophoresis, using bovine serum albumin, catalase, citrate synthase, egg albumin and aldolase as standards. Gel permeation chromatography on a Sephadex G-150 column indicated a similar molecular weight of 45 000, using aldolase, egg albumin, chymotrypsinogen and cytochrome *c* as standards.

The nature of the reaction catalyzed by the activating enzyme was first indicated by inhibitors. Most effective were the proteinase inhibitors chymostatin and antipain, as well as some selected proteins (Table III). This indicates that the activation is due to proteolysis. The inhibition by diisopropylfluorophosphate and phenylmethylsulfonyl fluoride together with the ineffectiveness of sulfhydryl reagents and complexing agents suggests that it might be a serine proteinase.

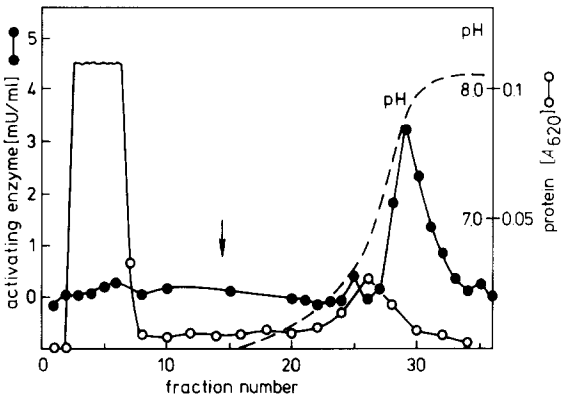

Fig. 3. Purification of the activating enzyme on a hemoglobin-Sepharose column. An aliquot (15 ml) from the pooled fractions (50 ml) from a DEAE-Sephacel column (Fig. 2) was applied at pH 6.0. At the arrow a gradient from pH 6.0 to 8.1 was started; fractions 27 to 33 were pooled.

The proteinase nature of the activating enzyme is further shown by its ability to liberate amino groups when incubated with casein, dimethylcasein and the insulin B-chain, proteins which inhibit IFP-synthase activation (Table III). When these proteins at a concentration of 50 μg/ml were incubated with 5 μg/ml of the purified activating enzyme under conditions used for the chromogenic proteinase substrates, a considerable increase in primary amino groups was observed. This effect was inhibited by chymostatin (about 50% inhibition at 2.0 μg/ml) and was most significant for the insulin B-chain. With the latter peptide the reaction was terminated after 2 h with about 0.8 amino groups liberated per molecule. As the fluorescamine reagent employed however differs considerably in effectiveness for various amino acids [11] these data can only be taken as approximate and as a first indication that peptide bonds are definitely split. These studies will be extended as they offer a simple way to investigate the sequence of amino acids required for the action of the enzyme.

As only a few proteins were found to be inhibitory (Table III) the peptide sequence necessary appears to be rather specific. It is therefore understandable that the purified activating enzyme did not split the following chromogenic or fluorogenic artificial substrates: Ala-, Glu-Phe-, carboxylpropyl-Phe-, *N*-benzoyl-Lys-, acetyl-Lys-, *N*-benzoyl-

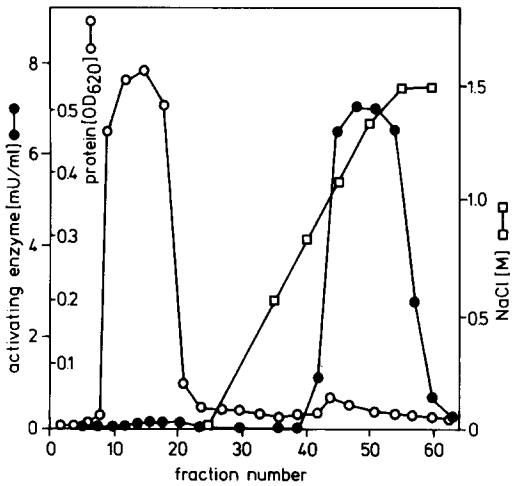

Fig. 4. Purification of the activating enzyme by affinity-chromatography on a fetuin-agarose column. Part of the pooled fractions (15 ml) from a DEAE-Sephacel column similar to Fig. 2 was applied and eluted with a 0–1.5 M NaCl gradient.

Tyr-, *N*-acetyl-Ala-, *N*-acetyl-Phe-, *N*-carbobenzoxy-Arg-, *S*-benzyl-Cys-*p*-nitroanilide. *N*-carbobenzoxy-Gly-nitrophenyl ester. Ala-Leu-, benzoyl-Arg-, Pro-naphthylamide. Tosyl-Gly-Pro-Arg-, *N*-carbobenzoxy-Gly-Pro-Arg-, *N*-acetyl-

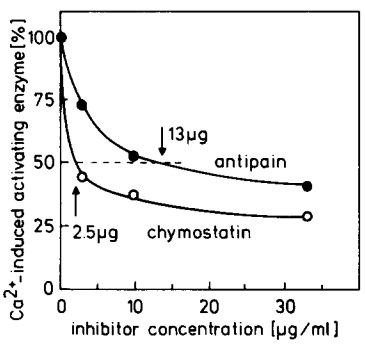

Fig. 5. Inhibition of the activating enzyme generated by Ca^{2+} in crude homogenates. The homogenate was prepared with EDTA from cells of standard volume as described in Materials and Methods. A concentrated solution of $CaCl_2$ was added to give a 2 mM final conc. and a $[Ca^{2+}]$ of 200 µM. The homogenate was incubated for 1 min at 25°C and aliquots of 1.5 ml were centrifuged for 30 s at 4°C and 12000× *g* in an Eppendorf centrifuge. The top 1 ml of the supernatants which also contains endogenous inactive IFP-synthase were pooled and used for the assay of activating enzyme without or with chymostatin (○) or antipain (●). Activating enzyme in the Ca^{2+}-sample was 8.1 mU/ml, whereas a control which was treated in parallel but not exposed to Ca^{2+} exhibited only 0.7 mU/ml.

TABLE IV

INHIBITION OF ACTIVATING ENZYME INDUCED BY CELL SHRINKAGE

A cell suspension (2 ml) was brought to the indicated osmotic values by addition of sucrose solution, aerated for 2 min and then homogenized. The crude homogenates were incubated at 25°C in the absence and presence of chymostatin (5 µg/ml) or antipain (25 µg/ml), and the activating enzyme was determined as described.

Osmotic value (mosmol/kg)	Activating enzyme (mU/ml) no inhibitor	Inhibition (%)	
		+ chymostatin	+ antipain
65 (control)	5.1	60	43
100	7.3	48	50
140	12.3	55	48
180	20.1	40	40
65 → 180 → 63 [a]	5.9	58	54

[a] After shrinkage for 2 min at 180 mosmol/kg the suspension was supplied with water to lower again the osmotic value to 63 mosmol/kg, and aerated for further 4 min.

Ala-Ala-Tyr-, *N*-acetyl-Lys-, *N*-succinyl-Ala-Ala-Phe-Amidomethylcoumarin.

In an attempt to show the action of the activating enzyme directly, we incubated it together with the most purified preparation of inactive IFP-synthase (Table I) under the conditions of the standard assay. At a time when maximum activation was observed (see Fig. 1) we recognized that on SDS-gel electrophoresis a certain band (molecular weight about 75 000) was greatly diminished. This observation again shows that the activating enzyme might be a proteinase. However the nature of this band remains unknown, as well as the position of the products. This is mainly due to the fact that the inactive IFP-synthase preparation available up to now is still rather impure. It gives several bands in the range where the purified active IFP-synthase (molecular weight about 70 000, Ref. 2) has to be assumed and the products of the proteinase reaction may thus be hidden under one of these impurities.

Activating enzyme in crude homogenates

It has been previously shown that an IFP-synthase activating enzyme can be generated from membranes in crude homogenates by addition of Ca^{2+} [5,6,15]. We can now show that this activa-

tion is inhibited by chymostatin and antipain (Fig. 5). We have previously also demonstrated [4,15] that only little activating enzyme can be found in crude homogenates from standard volume cells. When cells were shrunken for 2 min by a rise in the osmotic value this activity increased proportionally to the degree of shrinkage and was nullified shortly after cells were artificially reswollen by addition of water. We have now reinvestigated this observation to demonstrate that also this activity generated by the physiological stimulus in vivo can be inhibited by chymostatin and antipain (Table IV).

Discussion

Chromatography on immobilized proteins was of great help during the purification of the small amounts of protein associated with the activating enzyme. Hemoglobin-Sepharose, as a column material, was originally described for the purification of cathepsin D [14], whereas fetuin-agarose appears to have been used for the first time for the purification of a proteinase. Our results with the activating proteinase suggest that its binding to the two respective proteins might result from different types of interaction. Hemoglobin does not inhibit IFP-synthase activation (Table III) and the activating enzyme can be easily eluted from the respective column with a pH gradient at a low buffer concentration (Fig. 3). In contrast, fetuin was found to be a potent inhibitor (Table III) and elution occurred only at about 1 M sodium chloride (Fig. 4); a pH gradient similar to that used in Fig. 3 was not sufficient (data not shown). These observations might indicate that binding to fetuin could involve the active site of the proteinase. In contrast, the binding to the hemoglobin-Sepharose might be more unspecific and simply result from hydrophobic interaction. It appears of interest in this context that the inactive IFP-synthase is also retained on the hemoglobin-column (data not shown).

The inhibition of the purified activating enzyme by chymostatin, antipain and certain selected proteins (Table III), its ability to liberate amino groups from such proteins as well as the disappearance of a certain peptide band from the IFP-synthase preparation all indicate that it is a proteinase. This agrees with the previous observations that the activation of IFP-synthase in crude homogenates does not require any nucleotide or divalent ions [1,7] and can also be exerted by exogenous proteinases such as trypsin or chymotrypsin [7]. The fact that only few proteins can inhibit the action of the proteinase from *Poterioochromonas* on inactive IFP-synthase (Table III) together with the observation that all the artificial substrates used were not split, suggests that it requires a specific peptide sequence. Unfortunately we do not yet know whether the inactive IFP-synthase represents a zymogen from which a certain peptide sequence has to be split to render it active. Alternatively, it could be a complex with an inhibitory peptide which is removed by the action of the proteinase. The picture is even more complicated as other unphysiological ways to activate the IFP-synthase have been observed. One example is the reversible action of pyrophospahte ions (Table I), another the effect of cetyltrimethylammonium bromide which not only was found to stabilize the enzyme [2] but also shows a potency to activate it when added at pH 7.6 to a crude homogenate in the standard assay (data not shown). These substances may mimic other yet unknown physiological possibilities to regulate the IFP-synthase. This is reminiscent on other enzymes which can be activated by limited proteolysis and nevertheless show also regulation by allosteric effectors or covalent modification of another type. A well-known example from animals is the fructose bisphosphatase [16]. A more recent one from plants is the membrane bound 1,3-β-D-glucan synthase which can either be activated irreversibly by incubation with trypsin or reversibly by Ca^{2+} [17].

The recognition of inhibitors for the activating proteinase (Table III) allows the correlation of some older observations. In crude homogenates from standard volume cells an IFP-synthase activating enzyme is associated in an obviously inactive form with membranes of unknown identity and can be rendered active and soluble by Ca^{2+} [5,6,15]. This enzyme is inhibited by chymostatin and antipain similarly to the proteinase now purified (Fig. 5) and therefore appears to be identical or similar. Although it remains unclear at the moment how the proteinase is held cryptic and brought into action we can search now

more directly for the respective mechanism, for instance using antibodies. Furthermore, we could now show with the same inhibitors that the increase in IFP-synthase activating enzyme which results in vivo on cell shrinkage and disappears on cell swelling also might be a proteinase (Table IV). This indicates that the membrane-borne proteinase might indeed be part of the signal chain which is triggered by the physiological stimulus and ends up with isofloridoside phosphate- and isofloridoside-production to effect volume restoration.

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