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INTERACTION OF PROTEINASES AND THEIR INHIBITORS FROM YEAST

ACTIVATION OF CARBOXYPEPTIDASE Y

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Summary

In a crude extract of baker's yeast carboxypeptidase Y is predominantly found in an inactive form. A procedure for the isolation of the inactive form of the enzyme is presented. It is shown that the inactive form is identical to the reconstituted complex of carboxypeptide Y with its inhibitor. This complex is stable above pH 5, i.e., it remains inactive between pH 5 and 9. The conversion to the active enzyme occurs below pH 5, also in the absence of proteolytic enzymes. The inhibitor of carboxypeptidase Y can be removed enzymatically from the complex by treatment with proteinase B (EC 3.4.22.9) at pH 7. At pH 5, the carboxypeptidase Y-inhibitor complex is activated both by proteinase A (EC 3.4.23.6) and B.

Yeast proteinases are activated in a crude extract by incubation at pH 5 [3]. Based on the levels of proteinase A and B in an activated extract and on the time required for conversion to active carboxypeptidase Y, proteinase B is at least 10-times more effective than proteinase A. Peptides that arise during the pH 5-incubation procedure did not accelerate the proteolytic activation of carboxypeptidase Y.

The inhibitor of carboxypeptidase Y is completely degraded in the proteolytic activation steps, no accumulation of intermediates is observed. Only one form of active carboxypeptidase Y is found to be present in the proteolytically activated extracts, i.e., no polypeptide fragments of carboxypeptidase Y-inhibi-

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Abbreviations: Ac-Tyr-OEt, acetyltyrosine ethyl ester; Cbz-, benzyloxycarbonyl derivative of peptide.

tor remain bound to the enzyme after it has been activated by proteinase B. In vacuoles prepared from spheroplasts no inactive carboxypeptidase Y can be detected.

Introduction

Proteinases and their inhibitors in yeast have been the subject of many investigations and reviews [1,2]. Different proteolytic enzymes could be characterized [3—6] and specific inhibitors for each of these proteinases were isolated [7—11]. Whereas, the inhibitors of the endoproteinases, termed A and B, proved to be heat-stable proteins of a molecular weight less than 9000 [7,8,10] the inhibitor of carboxypeptidase Y turned out to be a rather high molecular weight protein (23 800) [9]. In this case a more detailed understanding of the function of the inhibitor protein could be reached. The observation that different activities of the exopeptidase were influenced to different degrees by the inhibitor gave rise to the notion of the inhibitor being a modifying protein [12].

The proteinases A and B and carboxypeptidase Y are localized in the vacuoles [13], whereas the inhibitors are found in the extravacuolar cytosol [2]. Proteinases are inhibited by formation of complexes with the inhibitors after homogenization of the yeast cell. The inhibition can be overcome by incubating a crude extract at pH 5 as originally described by Lenney [3,14]. During this activation step proteinase inhibitors are destroyed [5]. It seems plausible that the knowledge of the processes that occur during incubation at pH 5 will be of great help for an understanding of the physiological relevance of proteinases. For instance, the cascade mechanism that has been suggested to play a role in the activation of chitin synthase [1] could be studied with this system and thereby, assist the genetic approach [15] in trying to assign physiological functions to the proteolytic system of yeast.

As far as the activation of carboxypeptidase Y is concerned there are conflicting reports in the literature. Hayashi et al. [4], described an activation of what was then termed procarboxypeptidase Y by proteinase A. Barth et al. [12], could not detect any influence of a purified sample of proteinase A on the reconstituted carboxypeptidase Y-inhibitor complex. In order to allow for both results it was suggested that certain peptides by competing with the inhibitor of carboxypeptidase Y might enable proteinase A to catalyse the activation [12]. In this report it is shown that the inactive form of carboxypeptidase Y is identical to the reconstituted carboxypeptidase Y-inhibitor complex and that proteinase B is much more effective in activating carboxypeptidase Y than proteinase A. Evidence for a role of peptides in the proteolytic activation of the carboxypeptidase Y-inhibitor complex could not be detected.

Materials

Baker's yeast (Pleser Hefe, Darmstadt-Eberstadt) was obtained from BÄKO-Bäckereinkauf (Freiburg, F.R.G.). The peptides used were purchased from Bachem A.G. (Bubendorf, Switzerland). Carboxypeptidase Y was obtained

from Oriental Yeast Company (Tokyo, Japan), L-amino acid oxidase and horseradish peroxidase were from Sigma (Neubiberg, F.R.G.), alcohol dehydrogenase was purchased from Boehringer (Mannheim, F.R.G.). Dioxane was from Roth A.G. (Karlsruhe, F.R.G). Acetyltyrosine ethyl ester was a product of Sigma (Neubiberg, F.R.G.) as were the chemicals used in gel electrophoresis.

Ultrogel AcA 44 was a product of LKB (Brömma, Sweden); the ion exchange chromatography and affinity chromatography material were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals (reagent grade) were obtained from Merck A.G. (Darmstadt, F.R.G.). Purified carboxypeptidase Y-inhibitor was a gift of Dr. R. Barth, proteinases A and B were kindly supplied by Dr. F. Meussdoerffer and Dr. E. Kominami.

Methods

(1) Purification of the inactive form of carboxypeptidase Y. Step 1: 1 kg of baker's yeast was suspended in 1 l 100 mM Tris-HCl, pH 8.5, and homogenized about five times in the Manton-Gaulin-Homogenizer. The temperature was kept around 4°C throughout most of the procedures. Occasionally it rose to 8—10°C during homogenisation of the cells. After the first disruption solid Tris was added (6 g/l) to maintain the pH above 7.5. The material was then centrifuged at 10 000 × *g* for 60 min and filtered on Sephadex G-25 equilibrated with 50 mM Tris-HCl, pH 7.7 (standard buffer). Step 2: The protein fraction was mixed with 20 g preswollen DEAE-Sephadex A-50 per l and stirred for 60 min. The gel was poured into a column and washed with standard buffer. The activity was eluted with 0.3 N NaCl. Step 3: To the combined active fractions 390 g $(NH_4)_2SO_4$ were added per l. After removing the precipitate the $(NH_4)_2SO_4$ concentration was brought to 90% saturation by adding 277 g/l [4]. The pH was adjusted to 7 with 2 N NaOH. The precipitate was collected and desalted on a Sephadex G-25 column. This fraction (step 3) is referred to as crude preparation and used in incubation experiments described later. Step 4: The active fractions were then loaded onto a DEAE-Sephacel column that was equilibrated with the standard buffer. 50 ml of gel bed were used per l of crude extract. The activity was eluted with a linear KCl gradient (0—0.2 N). Step 5: The active pool was loaded on a 5 ml concanavalin A-Sepharose column that was equilibrated with the standard buffer substituted with 75 mM KCl/1 mM $CaCl_2$/1 mM $MnCl_2$. The column was washed with standard buffer, followed by 0.1 M potassium phosphate, pH 7.45. Active fractions were eluted with 1% α-methyl-D-mannoside in the phosphate buffer (Fig. 1). Step 6: The peak fractions were finally chromatographed on AcA 44 Ultrogel, equilibrated with degassed standard buffer. In this step the flow rate appeared critical and never exceeded 30 ml/h.

(2) Assay of carboxypeptidase Y. Carboxypeptidase Y was assayed with acetyltyrosine ethyl ester as substrate as described by Matern et al. [9]. The appearance of ethanol at pH 8, was determined with alcohol dehydrogenase at 360 nm. 1 unit of activity is defined as 1 μmol acetyltyrosine ethyl ester hydrolyzed per min. Peptidase activity was followed as outlined by Wolf and Weiser [15]. The liberation of the C-terminal amino acid was followed by its oxidation by L-amino acid oxidase at 405 nm. Proteinase A and B were assayed as described [3].

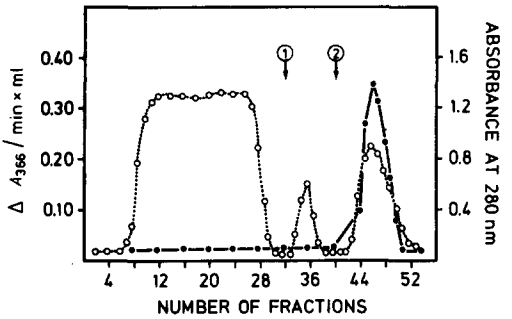

Fig. 1. Chromatography of the inactive form of carboxypeptidase Y on concanavalin A-Sepharose. The absorbance at 280 nm is plotted (o- - - - -o) along with the esterase activity of the samples after activation with dioxane (•———•). At the position indicated by 1 the column was washed with 0.1 M potassium phosphate buffer, pH 7.45. At the position 2 the elution with 1% α-methyl-D-mannoside was started.

(3) Dioxane treatment. Treatment of fractions with dioxane, to activate carboxypeptidase Y, was done by mixing 70 μl sample with 30 μl dioxane and incubating this mixture at 25°C for 10 min. Any precipitating material was removed by centrifugation for 4 min in a Hettich microfuge [4]. Higher concentrations of dioxane caused inactivation of carboxypeptidase Y. This could in part be prevented by adding *N*-Cbz-Phe-Leu, a substrate which is cleaved very rapidly by the enzyme [6]. Occasionally the dioxane treatment produced erratic data. This could always be prevented by using dioxane from a tightly closed bottle. We suspect, that peroxides interfered with the regular dioxane effect [16].

(4) Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed by the method of Laemmli [17]. The gels were run at 150 V for 5.5 h. Gel electrophoresis without SDS was performed on Pharmacia 4/30 gradient gels. The electrophoresis buffer was 0.09 M Tris-HCl/0.08 M boric acid/0.93 g/l Na_2EDTA, pH 8.4. The samples were run at 150 V for 16 h. Staining with 0.02% Coomassie blue was done overnight in 40% methanol/7% acetic acid. The gels were destained electrophoretically at 24 V. Activity in the gels was determined after keeping gel slices overnight in standard buffer at 4°C.

(5) Preparation of peptides from crude extract. Peptide fractions were prepared by incubating a crude extract at pH 5.1, in the presence of 0.02% NaN_3 and 0.2 M iodoacetamide (this inhibits peptidase activity of carboxypeptidase Y) for 24 h. The extract was filtered on a calibrated Sephadex G-50 column. The material that eluted between the positions of cytochrome *c* and NaCl was collected, loaded on a DEAE-Sephadex A-50 column (equilibrated with 20 mM Tris-HCl, pH 7.7), eluted with 0.4 NaCl, desalted on Sephadex G-25 and lyophilized. The powder was resuspended in water at 10 mg/ml.

(6) Incubation experiments. Incubations of crude extracts and purified preparations of the inactive form of carboxypeptidase were done in the presence of 0.02% NaN_3. Incubations at pH 5 were done by adjusting the pH with acetic acid or by dialyzing the sample for 4 h against 100-fold excess of 50 mM sodium acetate buffer. Proteinase B was applied in 0.1 M phosphate buffer, pH 7,

proteinase A was added in 0.1 M sodium acetate buffer, pH 5.5. At various times aliquots were assayed for carboxypeptidase Y activity.

(7) Preparation of a vacuole-containing fraction from spheroplasts. Spheroplasts of *Saccharomyces cerevisiae* were prepared and disrupted as described by Matern [18]. A vacuole-containing fraction was prepared as a pellet by centrifugation at 10 000 × *g* for 20 min. The pellet was resuspended in the original volume and homogenized in a standard tissue grinder.

(8) Protein determination. Protein was determined with the method of Lowry, using bovine serum albumin as standard [19].

Results and Discussion

Purification of the inactive form of carboxypeptidase Y

Inactive carboxypeptidase Y was isolated by a combination of ion exchange and affinity chromatography in addition to gel filtration and $(NH_4)_2SO_4$ precipitation. By starting the homogenization procedure in the buffer of pH 8.5, and adding solid Tris in the process, the actual pH never dropped below 7.5. As a result completely inactive carboxypeptidase Y was obtained in the supernatant of the crude extract (Table I). This confirmed the observations by Hayashi et al. [4] that keeping a crude extract at pH 8.5 increased the yield of inactive carboxypeptidase Y. A disturbing observation was the appearance of proteinase B activity in the eluate from DEAE-Sephadex (step 2) and the gel filtrate after $(NH_4)_2SO_4$ precipitation (step 3), whereas in the crude extract (step 1) no proteinase B activity was detected. This might, in part, be explained by the removal of free inhibitor of proteinase B through the chromatography on DEAE-Sephadex. The fact that, in the fractions with high proteinase B activity the highest amount of active carboxypeptidase Y, i.e., activity without dioxane activation, was found (data not shown) suggested an important role of proteinase B in the activation of carboxypeptidase Y.

TABLE I

PURIFICATION OF THE INACTIVE FORM OF CARBOXYPEPTIDASE Y

Esterase activity is defined as the difference between Ac-Tyr-OEt hydrolysis before and after treatment with dioxane.

Fraction	Volume (ml)	Esterase activity (units/ml)	Protein (mg/ml)	Specific esterase activity (units/mg)	Total esterase activity (units)	Purification factor (-fold)	Recovery (%)
1. Crude extract after gel filtration	1258	1.23	28.24	0.04	1548	1	100
2. DEAE-Sephadex eluate (0.3 NaCl)	281	2.04	8.19	0.25	573	6	37
3. $(NH_4)_2SO_4$ precipitate after gel filtration	210	2.44	3.31	0.74	512	18	32
4. DEAE-Sephacel eluate	142	2.63	1.74	1.51	373	38	24
5. Concanavalin A-Sepharose eluate	40	1.65	0.05	33.0	66	825	4
6. Ultrogel AcA 44 filtrate	122	0.52	0.008	65.0	63	1625	4

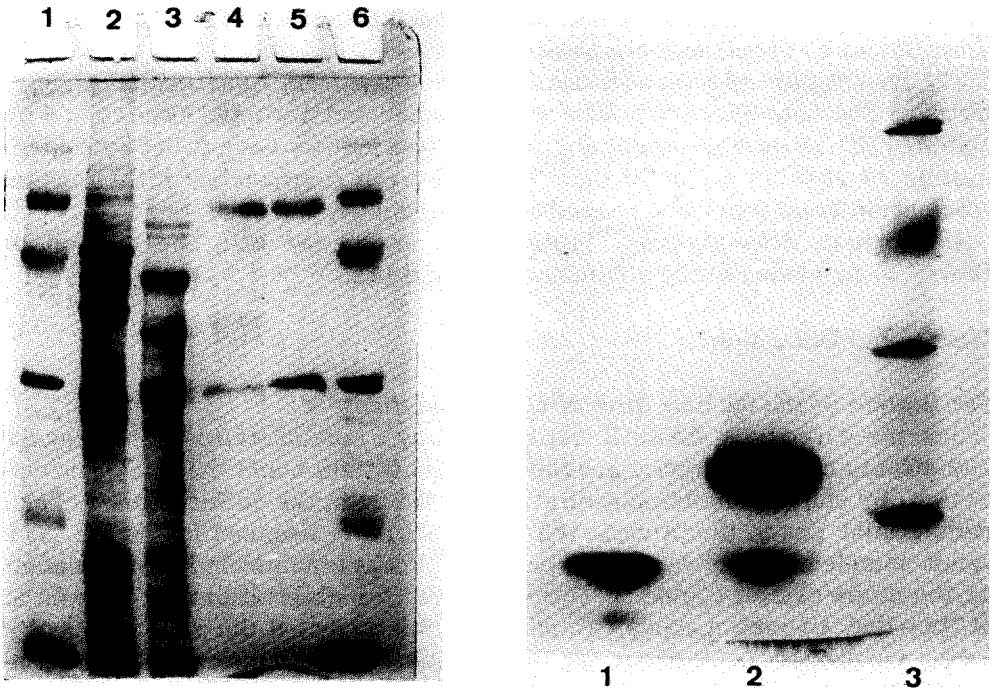

Fig. 2. SDS-polyacrylamide gel electrophoresis of various samples from the purification procedure listed in Table I. Slot 1 and 6: Standard proteins from top to bottom (molecular weights in brackets): phosphorylase *b* (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 000), α-lactalbumin (14 000). Slot 2: Crude extract (Table I, line 1). Slot 3: $(NH_4)_2SO_4$ precipitated protein (Table I, line 3). Slot 4: Sample after chromatography on concanavalin A-Sepharose (Table I, line 5). Slot 5: Purified inactive form of carboxypeptidase Y (Table I, line 6).

Fig. 3. Polyacrylamide gradient gels of the purified inactive form of carboxypeptidase Y and of pure active carboxypeptidase Y in the absence of SDS. Slot 1: Carboxypeptidase Y (25 μg). Slot 2: 50 μg purified inactive form of carboxypeptidase Y. Slot 3: Standard proteins from top to bottom (molecular weights in brackets): ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000), bovine serum albumin (67 000).

The appearance of various fractions during the purification procedure was monitored by gel electrophoresis as shown in Fig. 2. As may be seen from a comparison of slot 3 and 4 in Fig. 2, affinity chromatography on concanavalin A-Sepharose was the most efficient step of purification. This circumvented the repeated cycles of ion exchange chromatography originally used by Hayashi et al. [4].

In SDS gel electrophoresis the purified inactive form of carboxypeptidase Y gave rise to two bands of approx. molecular weights 60 000 and 30 000, respectively, under denaturating conditions (slot 5 in Fig. 2). In the absence of SDS (Fig. 3) one major band of molecular weight 86 000 was detected with a small extra band that comigrated with active carboxypeptidase Y (Fig. 3, slot 2). This was consistent with the observation that the purified inactive form of carboxypeptidase Y always exhibited a small esterase activity prior to activation with dioxane.

The molecular weight of the inactive form of carboxypeptidase Y was deter-

TABLE II

MOLECULAR WEIGHTS OF VARIOUS SAMPLES OF THE INACTIVE AND ACTIVE FORMS OF CARBOXYPEPTIDASE Y CALCULATED FROM MEASUREMENT WITH DIFFERENT METHODS

As standards the Pharmacia calibration kits for gel filtration or electrophoresis were used. The activity in the gel was determined after extracting gel slices in standard buffer. When two bands appeared two numbers are listed. n.d., not determined.

Sample	Gel filtration	SDS gel electrophoresis	Gel electrophoresis without SDS	
			Activity in gel slices	Stain with Coomassie blue
Carboxypeptidase Y	64 000 *	60 000	60 000	61 000
Inactive form of carboxypeptidase Y (Table I, step 6)	88 000 *	60 000 30 000	90 000	86 000
Inactive form of carboxypeptidase Y after dioxane treatment	65 000 **	n.d.	62 000	n.d.
Inactive form of carboxypeptidase Y after incubation with proteinase B	55 000 **	60 000	n.d.	62 000
Inhibitor of carboxypeptidase Y	23 800 ***	n.d.	n.d.	n.d.
Carboxypeptidase Y-inhibitor complex	n.d.	60 000 25 000	n.d.	86 000

* Gel filtration on Sephadex G-100.
** Gel filtration on Ultrogel AcA 44.
*** Data from Matern et al. [9].

mined under various conditions with different methods. The data for the carboxypeptidase Y-inhibitor complex are given by Matern et al. [9] and Barth et al. [12]. They are listed with the data for the inactive form of carboxypeptidase Y in Table II. From these results the conclusion can be drawn that inactive carboxypeptidase Y and the carboxypeptidase Y-inhibitor complex are identical. Further evidence in support of this hypothesis is derived from activation studies.

The inactive form of carboxypeptidase Y can be activated by treatment with dioxane as described in Methods. Not only the inactive form of carboxypeptidase Y, purified as summarized in Table I, but also the complex obtained by reassembling separately purified carboxypeptidase Y with its inhibitor, could be activated by dioxane treatment. The inhibitor protein isolated according to Matern et al. [9], could in turn reinhibit the inactive form of carboxypeptidase Y after it was activated by dioxane and after the denaturing agent was removed.

As shown by Matile and Wiemken [13], active carboxypeptidase Y is localized in the yeast vacuoles. According to Matern et al. [9], carboxypeptidase Y-inhibitor is found in the cytosol. It is conceivable that in addition to the complex of carboxypeptidase Y and its inhibitor there is another inactive form of the enzyme. In order to check this possibility, vacuoles and cytosol-containing fractions were prepared by gentle disruption of yeast spheroplasts, as described by Matern et al. [18]. As shown in Table III, the vacuolar fraction contained all carboxypeptidase Y activity and no additional carboxypeptidase Y activity appeared after treatment with dioxane. Also in the cytosol frac-

TABLE III

CARBOXYPEPTIDASE Y ACTIVITIES IN VACUOLES AND CYTOSOL-CONTAINING FRACTIONS DERIVED FROM SPHEROPLASTS

Fraction	Carboxypeptidase Y activity	
	without dioxane treatment (units/ml)	after dioxane treatment (units/ml)
10 000 × *g* pellet of disrupted spheroplasts (vacuolar fraction)	1.60	1.48
10 000 × *g* supernatant of disrupted spheroplasts (cytosol fraction)	<0.05	<0.05
Mixture (1:1) of pellet and supernatant	<0.05	1.51

tion dioxane treatment did not lead to the appearance of carboxypeptidase Y activity. Dioxane treatment of the combined fractions lead to the appearance of the same carboxypeptidase Y activity which had been measured in the isolated vacuolar fraction.

These observations allow the conclusion that all the inactive carboxypeptidase Y that can be detected in yeast extracts results from the combination of vacuolar carboxypeptidase and cytosolic carboxypeptidase Y-inhibitor after disruption of the yeast cells. This is in agreement with the experiments of Wiemken et al. [20], demonstrating the localization of more than 95% of the carboxypeptidase Y in vacuoles.

Proteinase catalyzed activation of the inactive form of carboxypeptidase Y

Fig. 4 demonstrates the ability of proteinase B to produce active carboxypeptidase Y from the inactive form of carboxypeptidase Y. That it is an effect of proteinase B itself, and not of impurities of the preparation, is demonstrated by the observation that in a control experiment addition of purified inhibitor of proteinase B completely prevented the activation. The recombined carboxypeptidase Y-inhibitor complex exhibits exactly the same kinetics of activation as does the inactive form of carboxypeptidase Y isolated from crude extract. Monitoring the activation of the inactive form of carboxypeptidase Y with SDS gel electrophoresis clearly shows that the effect of proteinase B is to remove the low molecular weight component of the complex, which was identified as the inhibitor of carboxypeptidase Y (data not shown).

The concentration of proteinase B (1 unit/ml) was selected to correspond to the activity found after 8 h of incubation of a crude extract at pH 5. If the amount of proteinase B was reduced to 0.5 units/ml, the rate of activation of the inactive form of carboxypeptidase Y was also reduced to less than 50% (cf. squares in Fig. 4). Free carboxypeptidase Y-inhibitor is inactivated by 0.5 units/ml of proteinase B about 10-times faster than the inactive form of carboxypeptidase Y (compare open circles with squares in Fig. 4). This confirms earlier observations by Barth et al. [12], that by interacting with its corresponding target proteinase carboxypeptidase Y-inhibitor is protected from proteolytic attack. The activating action of proteinase B on the inactive form of carboxypeptidase Y and on the recombined carboxypeptidase Y-inhibitor

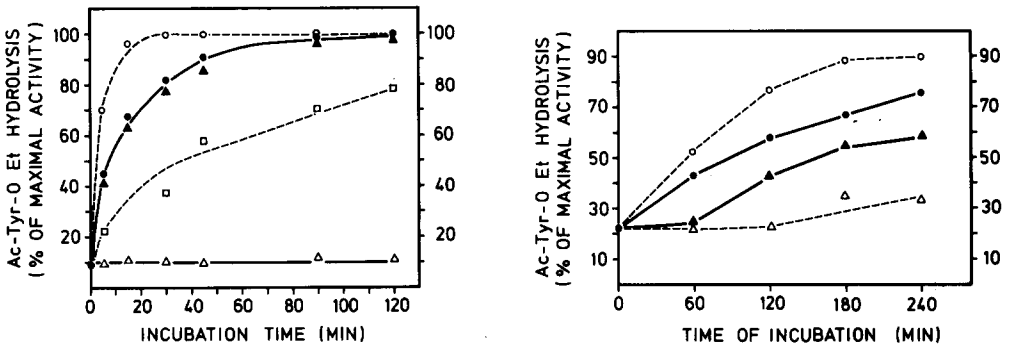

Fig. 4. Incubation of purified inactive form of carboxypeptidase Y, of reassembled carboxypeptidase A-inhibitor complex, and of free carboxypeptidase Y-inhibitor with proteinase B at pH 7. Purified inactive form of carboxypeptidase Y (●———●) and the recombined carboxypeptidase Y-inhibitor complex (▲———▲) were incubated with about 1 unit/ml proteinase B activity. Free carboxypeptidase Y-inhibitor was incubated with half the proteinase B activity (0.5 units/ml). For assay of carboxypeptidase Y-inhibitor, carboxypeptidase Y was added at the times of incubation indicated (○- - - - -○). The change in esterase activity upon incubating the inactive form of carboxypeptidase Y with 0.5 units/ml proteinase B is also shown (□- - - - -□). For each incubation a control was done with proteinase B-inhibitor 2 that inhibited azocoll hydrolysis, i.e. proteinase B activity, completely in the incubation mixtures. The control data varied only insignificantly (△———△). The notion 100% activity (maximal activity) refers to the activity without inhibitor in case of the complex. For the inactive carboxypeptidase Y it refers to the activity after treatment with dioxane.

Fig. 5. Incubation of the purified inactive form of carboxypeptidase Y with proteinase A at pH 5.0. The pH was adjusted by dialysis as described under Methods. Proteinase A was added so that the incubation mixture contained 20 units/ml. The incubations were done in the presence (○- - - - -○) and absence (●———●) of 15 mM Cbz-Phe-Leu. Incubation with 15 mM Cbz-Phe-Leu alone (without proteinase A) is also shown (▲———▲). If the inactive carboxypeptidase Y was incubated without proteinase A the data plotted as (△- - - - -△) were observed.

complex could be enhanced by lowering the pH from 7 to 5.

Following the incubation of the inactive form of carboxypeptidase Y with proteinase B on polyacrylamide gel electrophoresis in the absence of SDS did not show any band in addition to the ones that could be identified as the inactive form of carboxypeptidase Y and active carboxypeptidase Y. This suggests that immediately after the first proteolytic attack the inhibitor protein is completely removed from carboxypeptidase Y and no intermediates containing carboxypeptidase Y complexed with pieces of carboxypeptidase Y-inhibitor accumulate. Also during digestion of carboxypeptidase Y-inhibitor with proteinase B no appearance of intermediates was observed in SDS gel electrophoresis. The inhibitor (molecular weight according to Matern et al. [21], 23 000—24 000) was cut into peptides that were certainly smaller than molecular weight 6000.

If the inactive form of carboxypeptidase Y was incubated at pH 5.1 with concentrations of proteinase A that are present in activated crude extracts (6—8 units/ml), hardly any activation was observed. Even when purified proteinase A was used, at a concentration that corresponded to about 3-times the concentration present in an activated crude extract (20 units/ml), the activation remained small compared to the effects observed with proteinase B Fig. 5). From these data it seems reasonable to assume that proteinase A does not contribute significantly to the observed increase of carboxypeptidase Y

activity in crude extract incubated at pH 5. A likely role of proteinase A, however, might be to initiate a cascade by degrading the inhibitor of proteinase B and thereby producing active proteinase B, which in turn attacks inactive carboxypeptidase Y. A small but distinct increase in the rate of activation of the inactive form of carboxypeptidase Y caused by the addition of Cbz-Phe-Leu (open circles in Fig. 5) may be explained by the finding of Barth et al. [12], that this peptide renders carboxypeptidase Y-inhibitors bound to carboxypeptidase Y susceptible to proteolysis by trypsin. A similar though even smaller influence on the activation of carboxypeptidase Y was noted for an endogenous peptide fraction prepared from crude yeast extract (see Methods) used instead of Cbz-Phe-Leu. It seems, therefore, that formation of peptides during activation of crude extract by incubation at pH 5 does not play an important role in the activation of carboxypeptidase Y.

For the stability of the complex pH 5 appears to be a sharp turning point. Slightly above pH 5 inactive carboxypeptidase Y remains inactive even after 24 h of incubation. A little below pH 5 the complex was readily destabilized.

The activation of the inactive form of carboxypeptidase Y in a purified sample and in a crude extract resemble each other with respect to the participation of proteinases A and B (data not shown). The effect of the pH was different, however. The activation of purified inactive form of carboxypeptidase Y is summarized in Figs. 4 and 5. In the crude extract the highest rate of activation was achieved at pH 5, if the pH was adjusted by adding 30% acetic acid. If adjustment of pH was done by dialysis against 100-fold excess of 50 mM buffer, maximal rate of activation of carboxypeptidase Y was found at pH 6 (data not shown).

The crude preparation of the inactive form of carboxypeptidase Y (step 3 in Table I) no longer contained free inhibitors of proteinase A and B but still retained the proteinases A and B. When this fraction was incubated at pH 5 a lag time of about 10 h was observed before activation of carboxypeptidase Y (assayed by following hydrolysis of acetyltyrosine ethyl ester) occurred (cf. Fig. 6, unbroken line). When pepstatin A was added, a specific inhibitor of proteinase A, the lag time was increased to about 30 h. When the specific inhibitor

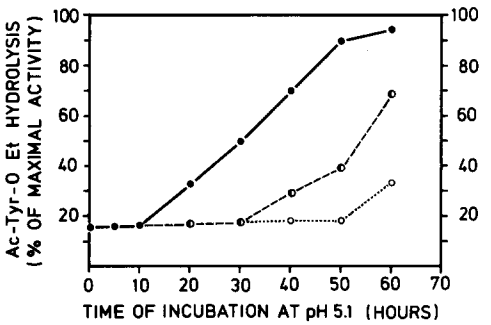

Fig. 6. Incubation at pH 5.1 of a crude preparation of the inactive form of carboxypeptidase Y (see step 3 in Table I). Esterase activity is plotted against the time of incubation. The results are shown for incubation without addition (●———●) and for incubations in the presence of either $1 \cdot 10^{-5}$ M pepstatin A (●------●) or 2 units/ml of proteinase B-inhibitor 2 (○-----○). The amount of proteinase B-inhibitor was sufficient to block the proteinase B activity in the incubation mixture for about 40 h.

of proteinase B was added, the lag time was even further increased (dotted line in Fig. 6). These results are in agreement with the kinetics of activation of carboxypeptidase Y obtained by incubating the purified inactive form of carboxypeptidase Y with proteinase A (Fig. 5) or proteinase B (Fig. 4), respectively. The results can be rationalized by assuming the cascade mechanism, suggested previously [1,5], along with the instability of the inactive form of carboxypeptidase Y at acid pH.

It was assumed by Barth et al. [12] that the peptides formed during autolysis at pH 5-incubation dissociate the inactive carboxypeptidase Y-inhibitor complex and thereby render the carboxypeptidase Y-inhibitor susceptible to proteolysis, i.e. inactivation by the proteinases A and B. The experiments leading to this hypothesis were done by studying the action of synthetic carbobenzoxy-*N*-dipeptides on incubations of complexes of carboxypeptidase Y with the carboxypeptidase Y-inhibitor in the presence of proteinases. To test this hypothesis experiments with the natural peptides prepared from the pH 5-incubated crude extract (see Methods) were done. No changes in the pattern of activation of carboxypeptidase Y were observed (data not shown). It therefore, remains open if the effects observed with the synthetic carbobenzoxy-*N*-peptides by Barth et al. [12] (which could be confirmed in our hands) have relevance to the processes that occur at the 'pH 5-activation' of crude extract. The most plausible explanation for the activation of carboxypeptidase Y, observed during incubation of a crude extract at pH 5, is that it results from the spontaneous self-activation of the inactive form of carboxypeptidase Y (which is identical with the carboxypeptidase Y-inhibitor complex) at a pH below 5.1, and also from the hydrolysis of the complex-bound carboxypeptidase Y-inhibitor by proteinase B which also leads to activation of carboxypeptidase Y (cf. Fig. 4). The proteinase B in turn may be activated from its inactive proteinase B-inhibitor complex by the proteinase B-inhibitor-hydrolyzing action of proteinase A which has been demonstrated previously [7]. Active proteinase A is formed from the proteinase A-inhibitor complex by lowering the pH to 5, as previously shown with purified preparations of carboxypeptidase A-inhibitor and proteinase A by Saheki et al. [8]. In summary, we consider the results described in the present paper as further evidence for the cascade mechanism of pH 5-activation of proteinases in crude extracts from yeast, as proposed previously [1,5].

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