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STUDIES ON THE CARBOXYPEPTIDASE Y-INHIBITOR COMPLEX OF YEAST

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Summary

We report in vitro studies on the interaction of several substrates with the carboxypeptidase Y-inhibitor complex of yeast. Inhibition of carboxypeptidase Y cleavage of two peptides by carboxypeptidase Y-inhibitor is shown to be competitive. The experiments show a wide variation in the degree of cleavage of a variety of peptide substrates by carboxypeptidase Y, despite the presence of the inhibitor protein.

The most likely explanation for this behaviour is a different capacity for the peptides to dissociate the inhibitor protein from the substrate-binding site of carboxypeptidase Y. While the carboxypeptidase Y-inhibitor is insensitive to proteolytic inactivation when complexed with carboxypeptidase Y, it is sensitive when in the free state. Addition of the substrate, *N*-Cbz-Phe-Leu, to the carboxypeptidase Y-inhibitor complex, however, allows proteolytic inactivation of the inhibitor protein. We suggest that the proteinase-inhibitor may play a crucial role in the regulation of proteinase activity.

The inhibitor protein generally protects proteins from unwanted proteinase action. However, it will allow cleavage of proteins which, by some signal triggered metabolically, become substrates due to the exposure of amino acid sequences normally buried, and exhibiting a high affinity for the proteinase.

Abbreviations used: Cbz-benzyloxycarbonyl-derivative of peptide; Ac-Tyr-O-Et, acetyl tyrosine ethyl ester.

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Introduction

Several intracellular proteolytic enzymes have been found in the yeast *Saccharomyces cerevisiae* [1]: Proteinase A [2,3], proteinase B [2,3], carboxypeptidase Y [4], carboxypeptidase S [5] and 3 aminopeptidases [6—8]. In addition 3 inhibitory proteins have been detected and characterized. One of these proteins specifically inhibits only proteinase A, the second only inhibits proteinase B and the third only inhibits carboxypeptidase Y [9—13]. The proteinases A, B and carboxypeptidase Y and their inhibitory proteins were found to be localized in different compartments of the cell. Whereas the proteolytic enzymes are localized in the vacuole of the cell, their respective inhibitors were found to reside in the cytoplasm [14—16]. There has been a lot of progress in the last 10 years in uncovering the proteolytic system of yeast. However, the functions of its components are still only poorly understood. The approaches that have been used to learn more about the role of the proteolytic system are: (1) purification of the components of the system and studying their effects in vitro and in vivo (for reviews, see refs. 1, 17—19); and (2) isolating mutants missing components of the proteolytic system and studying the link between the missing component and its consequence for cellular function [5,20—23].

This in vitro study of the carboxypeptidase Y-inhibitor complex was undertaken to evaluate the possible role of the proteinase inhibitors as regulators of proteinase activity.

Materials and Methods

Baker's yeast (Pleser Hefe, Darmstadt-Eberstadt) was obtained from BÄKO-Bäckereinkauf e.G.m.H. (Freiburg, Germany). The peptides used, were purchased from Bachem A.G. (Bubendorf, Switzerland). Carboxypeptidase Y was obtained from the Oriental Yeast Company (Tokyo, Japan), L-amino acid oxidase and horseradish peroxidase were from Sigma (Neubiberg, Germany), alcohol dehydrogenase was purchased from Boehringer (Mannheim, Germany), trypsin was obtained from Serva (Heidelberg, Germany). All other chemicals (reagent grade) were obtained from Merck A.G. (Darmstadt, Germany).

Crude extracts were prepared by using a French Pressure cell (Aminco, Silver Spring, Md., U.S.A.). Cells were suspended in 0.1 M potassium phosphate buffer (pH 7) in a ratio of 1 : 1 (w/v). Carboxypeptidase Y-inhibitor I^C was purified according to the method of Matern et al. [12]. Carboxypeptidase Y activity against Ac-Tyr-O-Et was measured as described by Matern et al. [12]: The appearance of ethanol at pH 8 was determined with alcohol dehydrogenase at 360 nm. One unit of activity is defined as μmol Ac-Tyr-O-Et hydrolyzed per min. Peptidase activity of carboxypeptidase Y was followed as outlined by Wolf and Weiser [5]: The liberation of the C-terminal amino acid was followed by its oxidation by L-amino acid oxidase at 405 nm. 1 unit is defined as nmol amino acid liberated per min. Inhibitory activity was defined as amounts of inhibitor required to inhibit a given unit of carboxypeptidase Y. Protein was determined by the method of Lowry et al. [24] using bovine serum albumin as standard. All measurements were done using one batch of carboxypeptidase Y-

and two batches of carboxypeptidase-inhibitor. No significant differences of the parameters measured were observed by using the two carboxypeptidase Y-inhibitor preparations. All values measured represent an average of at least five independent determinations.

Results

When testing the proteinase A and B activities in fresh, i.e. 'non-activated', crude extracts of yeast one measures only 40% of the proteinase A activity actually present [10] and no proteinase B activity [25]. This is due to inhibition of these proteolytic activities by the respective inhibitor proteins present in fresh crude extracts [9,10]. In the case of carboxypeptidase Y, inhibition of activity against 10 mM Ac-Tyr-O-Et by the endogenous inhibitor present in fresh crude extracts is 97% [12]. However, no inhibition by endogenous carboxypeptidase Y-inhibitor of carboxypeptidase Y activity was visible in fresh crude extracts when 7.5 mM of the peptide *N*-Cbz-Gly-Leu was used as substrate. This confirmed an earlier observation of Lenney [26] and indicated that different substrates of carboxypeptidase Y, at not very much differing molar concentrations, may have a very different influence on the degree of carboxypeptidase inhibition by a comparable amount of carboxypeptidase Y-inhibitor. This finding led to an investigation concerning the interaction of several *N*-benzyloxycarbonyldipeptides with carboxypeptidase Y and carboxypeptidase Y-inhibitor using the purified proteins. Table I shows the activity of carboxypeptidase Y against a variety of substrates and the extent of inhibition

TABLE I

PERCENT INHIBITION OF CARBOXYPEPTIDASE Y-ACTIVITY BY CARBOXYPEPTIDASE Y-INHIBITOR, CARBOXYPEPTIDASE Y-INHIBITOR-CONCENTRATIONS FOR HALF MAXIMAL INHIBITION AND SUBSTRATE CONCENTRATIONS FOR HALF MAXIMAL VELOCITY RATES OF CARBOXYPEPTIDASE Y IN THE PRESENCE OF DIFFERENT SUBSTRATES

All substrate concentrations were $1 \cdot 10^{-2}$ M in the tests, except *N*-Cbz-Phe-Leu, which because of decreased solubility was added in a concentration of $0.75 \cdot 10^{-2}$ M. Carboxypeptidase Y concentration was $1.49 \cdot 10^{-8}$ M and carboxypeptidase Y-inhibitor concentration was $1.46 \cdot 10^{-8}$ M and $7.3 \cdot 10^{-8}$ M, respectively. Buffer was 0.154 M $Na_4P_2O_7$, 0.04 M glycine (pH 8) in case of Ac-Tyr-O-Et hydrolysis and 0.2 M potassium phosphate (pH 7) in case of peptide cleavage measurements.

Substrate	% inhibition for		Concentration of carboxypeptidase Y-inhibitor necessary for 50% inhibition (M)	Substrate concentration of half-maximal velocity (M)
	Molar ratio enzyme : inhibitor of			
	1 : 1	1 : 5		
Cbz-Leu-Phe	4 ± 2	11 ± 3	$27.7 \cdot 10^{-8}$	$11.8 \cdot 10^{-4}$
Cbz-Phe-Met	2 ± 2	13 ± 3	$27.7 \cdot 10^{-8}$	$4.5 \cdot 10^{-4}$
Cbz-Phe-Leu	17 ± 5	23 ± 4	$10.9 \cdot 10^{-8}$	$1.3 \cdot 10^{-4}$
Cbz-Leu-Leu	13 ± 4	43 ± 2	$7.6 \cdot 10^{-8}$	—
Cbz-Ala-Phe	19 ± 6	48 ± 5	$6.6 \cdot 10^{-8}$	—
Cbz-Gly-Phe	23 ± 7	66 ± 8	$4.7 \cdot 10^{-8}$	$28.6 \cdot 10^{-4}$
Cbz-Gly-Leu	24 ± 2	80 ± 3	$4.0 \cdot 10^{-8}$	—
Cbz-His-Leu	37 ± 4	74 ± 7	$2.2 \cdot 10^{-8}$	—
Ac-Tyr-O-Et	85 ± 5	95 ± 5	$0.9 \cdot 10^{-8}$	—

by carboxypeptidase Y-inhibitor. The potential of carboxypeptidase Y-inhibitor to inhibit the cleavage of certain substrates by carboxypeptidase Y varies to a wide degree. Inhibition of Ac-Tyr-O-Et cleavage is greatest and inhibition of hydrolysis is also high for the peptide substrates *N*-Cbz-Gly-Phe, *N*-Cbz-Gly-Leu and *N*-Cbz-His-Leu. In contrast, carboxypeptidase Y cleavage of the peptide substrates *N*-Cbz-Leu-Phe or *N*-Cbz-Phe-Met is very little affected by the inhibitor protein. The approximate concentration of carboxypeptidase Y-inhibitor necessary for 50% inhibition of the carboxypeptidase Y cleavage of the various substrates tested, varies markedly. It ranges from an inhibitor: carboxypeptidase Y ratio of about 0.6 for Ac-Tyr-O-Et cleavage up to a ratio of about 19 for the cleavage of *N*-Cbz-Leu-Phe or *N*-Cbz-Phe-Met (Table I).

A possible explanation for the large variation in the amount of carboxypeptidase Y-inhibitor needed to prevent cleavage of the different peptides is that the peptides differ in their ability to dissociate the carboxypeptidase Y-inhibitor from the substrate binding site of carboxypeptidase Y. However, the data in Table I show that the apparent K_m values of four peptides investigated did not correspond to the ability of the peptide to release the enzyme from inhibition.

Lineweaver-Burk plots are only useful in describing proteinase-inhibitor interactions, if the inhibitor is mainly free [27]. Only in the presence of the two peptides *N*-Cbz-Leu-Phe and *N*-Cbz-Phe-Met is this requirement fulfilled satisfactorily for carboxypeptidase Y-inhibitor. Fig. 1 shows that inhibition by carboxypeptidase Y-inhibitor is in fact competitive in these cases.

Experiments described in the following section show that addition of *N*-Cbz-Phe-Leu to the carboxypeptidase Y-inhibitor complex not only activates carboxypeptidase Y as described above, but also renders the carboxypeptidase Y-inhibitor susceptible to proteolytic attack. Fig. 2 shows that incubation of carboxypeptidase Y-inhibitor at pH 5 or 7 leads to considerable loss of its inhibitory activity, probably due to physical denaturation. Addition of proteinase A (Fig. 2A), proteinase B (Fig. 2B) or trypsin (Fig. 2C) leads to an additional rapid inactivation of carboxypeptidase Y-inhibitor, due most likely to proteolysis. However, addition of carboxypeptidase Y completely protects

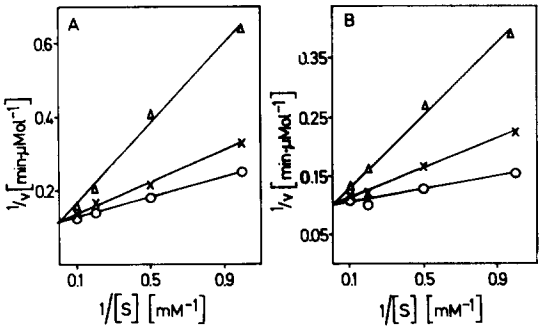

Fig. 1. Inhibition of carboxypeptidase Y cleavage of *N*-Cbz-Leu-Phe and *N*-Cbz-Phe-Met by carboxypeptidase Y-inhibitor (I^C). Plots are according to the method of Lineweaver and Burk [32]. A, *N*-Cbz-Leu-Phe; B, *N*-Cbz-Phe-Met. ○———○, $1.49 \cdot 10^{-8}$ M carboxypeptidase Y without addition; X———X, $1.49 \cdot 10^{-8}$ M carboxypeptidase Y with addition of $1.46 \cdot 10^{-8}$ M carboxypeptidase Y-inhibitor; △———△, $1.49 \cdot 10^{-8}$ M carboxypeptidase Y with addition of $7.3 \cdot 10^{-8}$ M carboxypeptidase Y-inhibitor. Buffers were the same as indicated in legend to Table I.

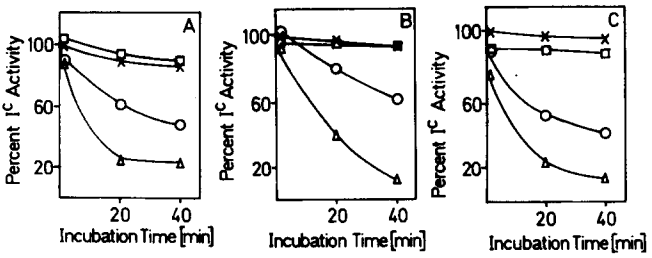

Fig. 2. Inactivation of carboxypeptidase Y-inhibitor (I^C) by physical denaturation and by proteinases. Its stabilization by carboxypeptidase Y. All incubations were performed in a volume of 0.1 ml at 25°C. A: Incubation of $29.7 \cdot 10^{-11}$ mol carboxypeptidase Y-inhibitor in 0.1 M sodium acetate pH 5. ○———○, without addition; X———X, with addition of $27.1 \cdot 10^{-11}$ mol carboxypeptidase Y; △———△, with addition of $22.1 \cdot 10^{-11}$ mol proteinase A; □———□, with addition of $27.0 \cdot 10^{-11}$ mol carboxypeptidase Y and $22.1 \cdot 10^{-11}$ mol proteinase A. B: Incubation of $29.7 \cdot 10^{-11}$ mol carboxypeptidase Y-inhibitor in 0.1 M potassium phosphate pH 7. ○———○, without addition; X———X, with addition of $16.2 \cdot 10^{-11}$ mol carboxypeptidase Y; △———△, with addition of $31.0 \cdot 10^{-11}$ mol proteinase B; □———□, with addition of $16.2 \cdot 10^{-11}$ mol carboxypeptidase Y and $31.0 \cdot 10^{-11}$ mol proteinase B. C: Incubation of $41.2 \cdot 10^{-11}$ mol carboxypeptidase Y-inhibitor in 0.1 M potassium phosphate pH 7. ○———○, without additoin; X———X, with addition of $27.1 \cdot 10^{-11}$ mol carboxypeptidase Y; △———△, with additoin of $41.2 \cdot 10^{-10}$ mol trypsin; □———□, with addition of $27.1 \cdot 10^{-11}$ mol carboxypeptidase Y and $41.2 \cdot 10^{-10}$ mol trypsin. In the case of incubation C $47.2 \cdot 10^{-10}$ mol of soya bean trypsin inhibitor were added after incubation to eliminate tryptic activity in the Ac-Tyr-O-Et test. Aliquots of 0.03 ml of the incubation mixtures were tested for activity.

carboxypeptidase Y-inhibitor from physical denaturation and surprisingly also from inactivation by proteinases A and B and trypsin (Fig. 2). Thus formation of the proteinase-inhibitor complex seems to protect the carboxypeptidase Y-inhibitor from proteolytic inactivation. This result seems to be contradictory to the observed inactivation of carboxypeptidase Y-inhibitor in crude extracts, where carboxypeptidase Y is present to form the inhibitor complex [28]. However, a possible explanation of the conflicting results was found when the carboxypeptidase Y-inhibitor complex was treated with trypsin in the presence of a peptide like *N*-Cbz-Phe-Leu. Increasing concentrations of the peptide (3.75, 7.5 and 12.75 mM) led to a progressive inactivation by trypsin of carboxypeptidase Y-inhibitor of 11, 25 and 41% of its original activity within 30 min. This observation strongly supports the idea that *N*-Cbz-Phe-Leu displaces the inhibitor from the peptide binding site of carboxypeptidase Y thereby leading to a conformationally altered or a free inhibitor protein that is now accessible to proteolytic inactivation. Thus, the displacement of the carboxypeptidase Y-inhibitor from the carboxypeptidase Y-binding site by peptides that are present in the crude extract seems to be the event that triggers subsequent proteolytic inactivation of the inhibitor.

Discussion

There is very little known about the regulation of proteolysis in vivo [29,30]. Proteinase activity, substrate accessibility and interactions between the proteinase bearing cell compartment and the substrate containing compartment have to be taken into account. Several authors have proposed conformational changes of proteins resulting from removal of a coenzyme or binding of a metabolite as the trigger to specifically render these proteins susceptible to

proteolysis [29,31]. This new susceptibility arises presumably from the exposure of amino acid sequences formerly buried, and which are 'good substrates' for the proteinase. The experiments with carboxypeptidase Y-inhibitor complex described above show the highly differing potentials of substrates for proteinase cleavage despite the presence of the inhibitor protein and we suggest this is probably due to dissociation of the complex. This finding is consistent with the following extension of the model for the regulation of cytosolar appearing proteinase activity cited above. At some signal, newly exposed amino acid sequences of a protein are able to bind more tightly to the proteinase, causing the dissociation of the proteinase inhibitor from the substrate binding site and thereby permitting proteolytic cleavage of the substrate. Thus the substrate itself controls proteolysis at whenever and wherever it may appear in the cell. This varies from the model in which substrate specificity controls proteolysis in that the proteinase inhibitor is involved as a second element to lower the apparent 'sensitivity' of the proteinase against proteins in general, but enhancing the 'specificity' for the cleavage of proteins that become substrates at some signal.

In addition, dissociation of the proteinase-inhibitor complex might be facilitated by some signal, such as a change in pH, as was shown for the partial dissociation of the proteinase A-inhibitor complex in crude extracts [10,18]. Furthermore, it had been shown that proteinases A, B and carboxypeptidase Y can be activated in their complexes in vitro by a proteolytic cross inactivation of their respective inhibitors [10,18]. Experiments with the carboxypeptidase Y-inhibitor complex described in this work show that proteolytic inactivation of the inhibitor protein is only possible after its dissociation from the substrate-binding site of the proteinase by a substrate. This possibility might also be important as a regulatory mechanism in vivo and lead to locally active proteinase, where an appropriate substrate has to be cleaved. Experiments concerning the dissociation of the inhibitor complexes of proteinases A and B are under way.

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