CHITIN SYNTHETASE ACTIVATING FACTOR FROM YEAST, A PROTEASE

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SUMMARY

The neutral proteolytic activity present in preparations of chitin synthetase activating factor (AF) from yeast and measured with "Azocoll" as substrate, is completely inhibitable by the heat-stable protein inhibitor of AF. The effects of the inhibitor on the chitin synthetase activating system and on the proteolytic reaction are quantitatively identical. It is concluded that AF is very probably a protease. These and other results point to a notable similarity between AF and its inhibitor, the proteinase and inhibitor reported by Lenney and Dalbec ((1969) Arch. Biochem. Biophys. 129, 407) and the tryptophan synthetase "inactivating factor" and "protecting factor" reported by Manney ((1968) J. Bacteriol. 96, 403).

It has been found recently (1) that yeast chitin synthetase, a particulate enzymatic system, can be isolated in an inactive or zymogen state. Incubation with a factor extracted from yeast (activating factor, AF[1]) or with trypsin leads to activation of the zymogen. This result suggested that AF, which has been recovered in the protease-rich vacuole fraction of a protoplast lysate (2), might also be a proteolytic enzyme. It was of interest to confirm this hypothesis, both because of its intrinsic importance and of the recent publication of several reports dealing with systems from yeast which bear an apparent resemblance to AF and its heat-stable protein inhibitor (1). A component of one of these systems has been characterized as a protease (3) and that of another one, the "inactivating factor" of tryptophan synthetase (4) may also be a proteolytic enzyme (5).

METHODS

Chitin synthetase zymogen and activating factor inhibitor were prepared from Saccharomyces cerevisiae S288C as previously communicated (1,6,7). AF

[1] The abbreviations used are: AF, chitin synthetase activating factor; BAEE, benzoylarginine ethyl ester; PCMB, p-chloromercuribenzoate; PMSF, phenylmethylsulfonyl chloride; DFP, diisopropylfluorophosphate.

was obtained by sonic extraction of a vacuole fraction from S. cerevisiae X2180 (2). Preparations obtained according to Cabib and Farkas (1) yielded similar results. Zymogen and AF were measured as described earlier (1,7). Azocoll, an insoluble protease substrate consisting of cowhide protein with an included red dye, was obtained from Calbiochem. Proteolytic activity on Azocoll was measured by incubating substrate and enzyme at 30° with 0.1 M imidazole chloride at pH 7.4, followed by filtration of the reaction mixture and measurement of the absorbance of the filtrate at 520 nm. BAEE hydrolysis was measured in the presence of the same buffer, from the change in absorbance at 253 nm (8). Bovine Achilles tendon collagen was purchased from Worthington and denatured by heating at 60-70°. Proteolytic activity on this substrate was determined after incubation at 30° by precipitating out the remaining protein with 5% perchloric acid and measuring the absorbance of the supernatant fluid at 215 and 225 nm (9).

RESULTS

As will be reported elsewhere (2) both the AF activity and the proteolytic activity with Azocoll as substrate are enriched in about the same proportion in a vacuole fraction isolated from yeast. Moreover, the highly purified protein inhibitor of AF (1) also inhibits the Azocoll protease in the extract (Fig. 1). By increasing the amount of inhibitor, the activity can be practically abolished. When the effect of the inhibitor was measured in parallel experiments on the activation of chitin synthetase and on Azocoll hydrolysis, the inhibition curves were practically superimposable (Fig. 2.). These results strongly support the conclusion that the activating effect on zymogen and the proteolytic activity are manifestations of the same enzyme.

Since Azocoll consists of a mixture of proteins and the dye it contains is not covalently attached to the protein, some experiments were repeated using purified denatured collagen as substrate and measuring the liberated peptides from their ultraviolet absorption. The results were similar to those obtained with Azocoll, except that the maximal inhibition achieved with excess

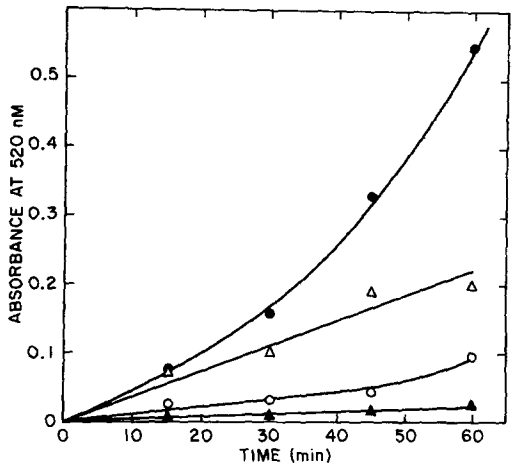

FIG. 1. Effect of heat-stable inhibitory protein on the AF activity on Azocoll. All assays contained 0.45 μg of AF protein. The amount of inhibitor added was none (●), 20 ng (△), 33 ng (○) and 60 ng (▲). Total volume of the reaction mixture was 0.3 ml. The exponential shape of the time curve is unexplained. Trypsin yields a similar curve. However, graphs of activity as a function of amount of enzyme added were linear in both cases.

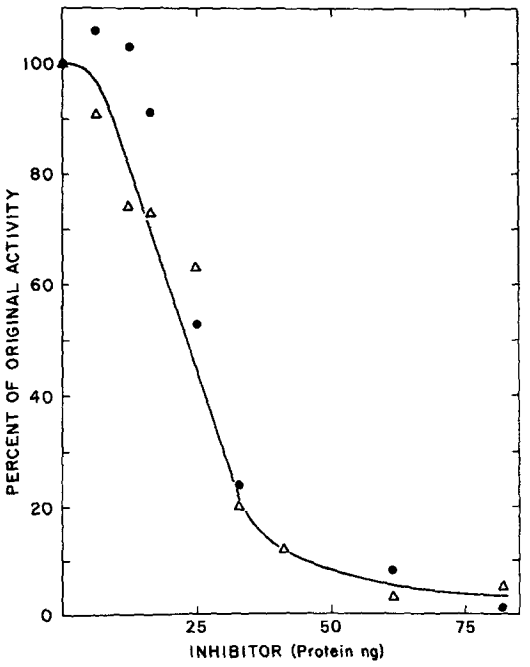

FIG. 2. Titration of AF with inhibitor in both chitin synthetase (●) or Azocoll (△) assay. In the former determination the incubation time with AF was 30 min, in the latter 60 min. The same preparation of AF was used in both experiments, using 0.54 μg, *as* protein, in each assay.

inhibitor was 80%.

In view of the similar effect of trypsin and AF on zymogen, it was of interest to compare these two activities with different proteolytic substrates. For this purpose, amounts of both enzymes which showed the same activity on Azocoll were allowed to act on BAEE (Table I). The effect of AF on the latter

TABLE I. Different specificity of AF and trypsin

	Substrate	
	Azocoll	BAEE[a]
	ΔA_{520}/hr	ΔA_{253}/hr
Trypsin (0.3 μg)	0.285	0.5
AF (0.44 μg)	0.285	0.027
AF (0.44 μg) + inhibitor (43 ng)	0.03	0.015

[a] In order to obtain measurable rates with this substrate larger amounts of AF than shown were employed. The rate was recalculated for 0.44 μg of AF, as used with Azocoll.

substrate was 20 times smaller than that of trypsin. The difference is probably even greater, because the activity of AF on BAEE is inhibited only 45% by the heat-stable inhibitor, against a value of 90% inhibition for the Azocoll reaction (Table I). Thus, AF and trypsin appear to have a different specificity, a conclusion which can also be inferred from the fact that trypsin digests the AF protein inhibitor (6), whereas AF is inhibited by it. Conversely, AF was not inhibited by soybean trypsin inhibitor (not shown).

The activity of AF on Azocoll was inhibited by PCMB and by PMSF. The concentrations for 50% inhibition were 30 μM and 6.5 μM, respectively.

DISCUSSION

The proteolytic nature of AF, suggested by the analogous effect of trypsin and AF on the chitin synthetase zymogen, has been confirmed by the similar enrichment of chitin synthetase activating factor and protease activity

in the vacuole fraction (2) and especially by the identical titration curve of both activities with the highly purified AF inhibitor. This finding has greatly simplified the assay for both AF and inhibitor, which can be carried out by the protease reaction, rather than using the more complicated chitin synthetase system (1,7). This assay is currently used in further studies with the inhibitor.[2] Another area of potential application is protection of yeast enzymes from proteolysis in crude extracts, a troublesome problem in enzyme purification (10). Insofar as Azocoll hydrolysis is representative of proteolytic action at neutral pH, protection could be afforded by the addition of sufficient heat-stable inhibitor, which by virtue of its specificity, would be potentially less harmful to other enzymes than the commonly used DFP or PMSF (6). Other proteolytic activities, however, are not sensitive to the heat-stable inhibitor. Such is the case with an activity measured at pH 3.6 with hemoglobin as substrate (2). Indeed, several proteolytic activities have been identified in yeast (11,12). The properties of AF resemble closely those of the so-called proteinase B (11). Both are inhibited by PCMB and by serine reagents such as DFP or PMSF. More important, Lenney and Dalbec (3) have reported that proteinase B can be isolated as an inactive complex with an inhibitor which is a heat-stable, small molecular weight protein, and reacts stoichiometrically with the protease. The analogy with AF and its inhibitor is striking but further work will be necessary to decide on the identity of the two activities.

A close parallel can also be drawn between AF and the "tryptophan synthetase inactivating factor" found by Manney in yeast (4) and recently purified by Katsunuma et al. (5). This factor resembles AF in that it is present in higher amounts in cells grown in minimal than in rich medium (1,4) and in cells from stationary phase as compared to those from logarithmic phase (1,5). Here, too, factor activity is blocked by a heat-stable inhibitor, which is eluted from Sephadex G-25 within the void volume. Katsunuma et al.

[2] R. Ulane and E. Cabib, unpublished work.

(5) consider that the factor or factors (two activities were separated during purification) is probably a protease.

The different circumstances under which each factor was discovered led us to call ours "activating factor" and Manney to call his "inactivating factor." More work is needed to decide whether any or both of these names designate the correct physiological function.

Finally, discussions about the regulatory significance of these systems (5) should take into account the fact that enzyme and inhibitor may be compartmentalized in the cell, as has been shown to be the case for AF and its inhibitor (1,2). This situation will also affect the determination of yeast proteases, when the methods used in cell extraction lead to the disruption of internal organelles. The activity measured in this case will not be a direct reflection of that present in the cell, but of the relative amounts of enzyme and inhibitor.

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