Biochimica et Biophysica Acta, 525 (1978) 338—345

BBA 68493

PURIFICATION AND PROPERTIES OF AN INHIBITORY PROTEIN OF CHITIN SYNTHETASE FROM *MUCOR ROUXII*

EVERARDO LOPEZ-ROMERO *, JOSE RUIZ-HERRERA and SALOMON BARTNICKI-GARCIA **

Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas and Departamento de Genética y Biología Molecular, Centro de Investigación y Estudios Avanzados, Instituto Politécnico Nacional, México, D.F. (Mexico) and Department of Plant Pathology, University of California, Riverside, Calif. 92521 (U.S.A.)

(Received January 27th, 1978)

Summary

A soluble protein inhibitor of chitin synthetase (UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxyglucosyltransferase, EC 2.4.1.16) was isolated from the cytoplasm of *Mucor rouxii*. By gel filtration, the molecular weight of the inhibitor was estimated to be 17 500. The inhibitor was effective against crude or purified (chitosome) preparations of chitin synthetase. Unlike the chitin synthesis inhibitor from *Saccharomyces* spp., the inhibitor from *M. rouxii* does not operate by blocking the proteolytic activation of chitin synthetase zymogen but by inhibiting the operation of activated enzyme. Presumably, the inhibitor forms part of the regulatory mechanism of chitin synthesis in the cell.

Introduction

Cabib and Farkas [1] proposed a model for the activation of chitin synthetase (UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxyglucosyltransferase, EC 2.4.1.16) "zymogen" from *Saccharomyces* spp. by an activating factor later identified as proteinase B [2,3]. This model includes a heat-stable protein [4] which inhibits the protease [5]. It was suggested [1] that this protein inhibitor served as a safety mechanism to prevent untimely initiation of chitin synthesis.

* Predoctoral fellow from CONACYT, Mexico.
** To whom reprint requests should be sent.

The mycelial form of *Mucor rouxii* contains a soluble non-dialyzable inhibitor of chitin synthesis [6]. Yeast cells of *M. rouxii* also contain the inhibitor and in greater concentration than the mycelium [7]. In this communication, we describe the purification and properties of the inhibitory protein of chitin synthetase from yeast cells of *M. rouxii*. The inhibitor was tested for its ability to block the activity of crude or purified preparations of chitin synthetase. The crude preparation was the mixed membrane fraction of a cell-free extract, treated with a detergent (Brij 36T) to prevent spontaneous activation of the zymogen [7]. The purified enzyme preparation was a suspension of chitosomes; these newly found microvesicle-like structures are considered to be the cytoplasmic containers and conveyors of chitin synthetase zymogen in fungal cells [8,9].

Methods

The strain of *Mucor rouxii*, the culture media and growth conditions have been described [7]. Yeast cells were harvested by filtration through a sintered glass filter, washed with 0.05 M KH_2PO_4/NaOH buffer, pH 6.0, containing 10 mM $MgCl_2$ (referred to throughout this publication as phosphate/magnesium buffer) and broken with a Braun MSK cell homogenizer. Cell-free extracts were centrifuged at 1000 $\times g$ to remove cell walls and the supernatant was centrifuged at 68 000 $\times g$ for 1 h. The pellet was labelled mixed membrane fraction. The supernatant fraction was used as starting material for the purification of the inhibitor. Chitosomes from yeast cells of *M. rouxii* were purified by a sucrose gradient centrifugation method [9]. Chitosomes from mycelium of *M. rouxii* were prepared by the same technique.

Mixed membrane fraction treated with the non-ionic detergent Brij 36T was used to measure the inhibition of activation of chitin synthetase during purification of the inhibitor. Mixed membrane fraction was resuspended in phosphate/magnesium buffer, treated with 10 mM Brij 36T for 1 h at 0°C, centrifuged at 68 000 $\times g$ for 1 h and washed twice by centrifugation at the same speed. The sediment was resuspended in buffer and kept at 0°C. This preparation was stable and could be activated by incubation with proteases [7].

Unless otherwise stated, inhibitor assays were performed as follows: Brij-treated mixed membrane fraction was incubated in a final volume of 125 μl with 20 mM *N*-acetylglucosamine, 0.2 mM ATP, 0.5 mM UDP-[^{14}C]GlcNAc (0.2 Ci/mol), 10 mM $MgCl_2$ and 50 mM KH_2PO_4/NaOH buffer, pH 6.0, in the presence of trypsin (400 μg/ml) or Rennilase (6.6 mg/ml) and several dilutions of inhibitor for 30 min at 22°C. These concentrations of proteases gave optimum activation (10—15-fold) of Brij-treated mixed membrane fraction. In some assays, suspensions of purified chitosomes were used in lieu of mixed membrane fraction as a source of chitin synthetase. In such cases, much lower concentrations of trypsin or Rennilase were employed. The reaction was stopped with a drop of glacial acetic acid and chitin synthesized was measured by a filtration method [7]. 1 unit of chitin synthetase was the amount of enzyme that catalyzed the incorporation of 1 nmol of *N*-acetylglucosamine into chitin in 1 min. Inhibitor activity was measured as percentage of inhibition of chitin synthetase. Inhibitor activity was also expressed in units; one unit being that

amount which caused 1% inhibition. Specific activity was calculated per mg protein. The relative amounts of chitin synthetase and inhibitor in the assays were adjusted so that percentage of inhibition was in the range of 10—50%, where inhibition was fairly proportional to inhibitor concentration. Proteolytic activity was measured with either casein (pH 6.0) or acid-denatured haemoglobin (pH 3.0) [10]. After 1 h at 22°C, the mixtures were precipitated with 0.3 M trichloroacetic acid, and after 1 h at 0°C they were centrifuged at 1000 × *g*. The supernatant was assayed with Folin's reagent [11]. Neutral proteinase activity was also measured with Azocoll (Calbiochem, La Jolla, California, U.S.A.) in 0.1 M Tris · HCl buffer at pH 7.4; acid protease was also measured with fibrin blue (Calbiochem, La Jolla, Calif.) in 0.1 M citrate buffer, pH 2.5. Protein was measured by Lowry's method [12]. Molecular weight determinations were made by gel filtration on columns of Sephadex G-100 (Pharmacia, Uppsala, Sweden) and Bio-Gel P-60 (BioRad, Richmond, Calif.). The columns were calibrated for each different concentration of NaCl used in the eluting buffer against a set of non-enzymatic protein markers (myoglobin, chymotrypsinogen, ovalbumin and bovine albumin) purchased from Schwarz/Mann (Orangeburg, N.Y.).

Trypsin, 5 × crystallised and casein (Hammersten) were from NBCo (Cleveland, Ohio); a pure acid protease from *Rhizopus chinensis* was obtained from Miles Laboratories (Elkhart, Ind.). Rennilase, a crude acid protease from *Mucor miehei*, was supplied by Novo Enzyme Corp. (Mamaroneck, N.Y.). Mexicain a crystalline protease from *Pileus mexicanus* [13], was a gift from M. Castañeda-Agulló, Instituto Politécnico Nacional, México. Brij 36T, a product of Atlas de México, was a gift from Carlos Gitler, Instituto Politécnico Nacional, México. Neutral protease from *Phycomyces blakeesleanus* was provided by P. Fischer, California Institute of Technology, Pasadena. Crude bovine haemoglobin was purchased from Calbiochem. UDP-[1-^{14}C]GlcNAc was obtained from ICN (Irvine, Calif.). Sephadex gels G-25 and G-100 were from Pharmacia (Uppsala, Sweden). Bio-Gel A-5m was from BioRad (Richmond, Calif.). Soybean trypsin inhibitor was from Sigma Chemical Co. (St. Louis, Mo.). Pepstatin A was from the Protein Research Foundation (Osaka, Japan).

Results

Purification of the inhibitor

In the studies by Cabin and co-workers [1,4,5], the protein inhibitor of chitin synthesis was extracted from *Saccharomyces* cells by boiling. This method was not applied to isolate the inhibitor from *M. rouxii* since heating at 70—100°C completely abolished the inhibitory properties of the supernatant fraction. Instead, we precipitated the inhibitory substance from the supernatant fraction by 0.4 saturation with $(NH_4)_2SO_4$ (a larger quantity of inhibitor could be precipitated by 0.7 saturation but the specific inhibitory activity of the precipitate was lower and its protease content higher). The ammonium sulfate-precipitated fraction was dialyzed and subjected to gel filtration in a Sephadex G-100 column (Fig. 1). Fractions 45—72 were combined, concentrated in an Amicon Model 202 ultrafiltration cell equipped with a UM-2 membrane (Amicon, Lexington, Massachusetts) and further fractionated by gel filtra-

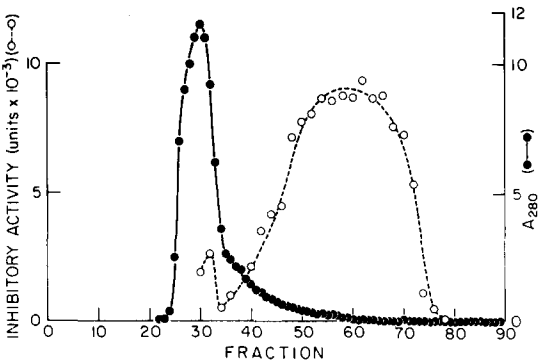

Fig. 1. Separation of chitin synthetase inhibitor by gel filtration. The crude supernatant was precipitated at 0.4 saturation with ammonium sulfate; the precipitate was redissolved in phosphate magnesium buffer, pH 6.0, dialyzed against the same buffer, applied (20 ml; 350 mg protein) to a Sephadex G-100 column (83 X 2.6 cm) and eluted with the above buffer. Fractions of 5 ml were collected. Absorbance was recorded at 280 nm; inhibitory activity is expressed in units (see Methods).

tion on Sephadex G-25 (Fig. 2). The inhibitor appeared after the void volume. Active samples (53—74) were combined and concentrated. Representative data for the preparation of one batch of inhibitor are shown in Table I. The data in Figs. 1 and 2 correspond to another batch of the inhibitor. The yields of inhibitor were variable.

The Sephadex columns separated the inhibitor from the rest of the protein found in the ammonium sulfate fraction (Figs. 1, 2; A_{280} determinations). The separation was probably due to some weak binding between the inhibitor and the Sephadex gel which caused sufficient retardation in the movement of the inhibitor through the columns. There was no peak of ultraviolet light absorbance corresponding to the inhibitor peak in the gel filtration profiles (Figs. 1 and 2) because the amount of inhibitor present was too small to be detected spectrophotometrically. The inhibitor yield in the preparation shown in Fig. 2 was 730 μg protein as measured by Lowry's method. In both Sephadex G-100 and Sephadex G-25 separations, a small portion of the inhibitory material

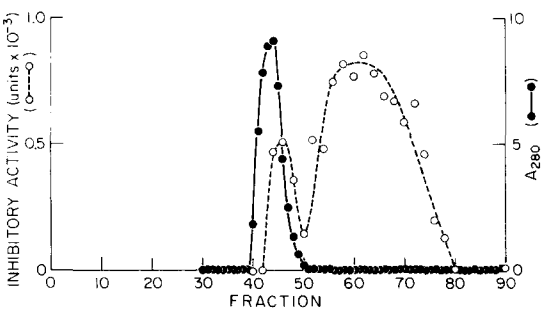

Fig. 2. Purification of chitin synthetase inhibitor by gel filtration. The inhibitor separated by gel filtration in Sephadex G-100 (Fractions 45-72; Fig. 1) was concentrated (23 ml; 31 mg protein) and applied to a column of Sephadex G-25 (100 X 2.5 cm) and eluted with phosphate/magnesium buffer. Fractions of 5 ml were collected. Absorbance was measured at 280 nm; inhibitory activity was measured and expressed in units.

TABLE I

PURIFICATION OF CHITIN SYNTHETASE INHIBITOR FROM CYTOSOL OF *MUCOR ROUXII*

The same batch of Brij-treated mixed membrane fraction was used for assays at all steps of purification.

Step	Total protein (mg)	Total activity * (units)	Specific acitivity (units/mg protein)	Yield (%)	Purification factor (-fold)
Crude supernatant fraction	1 532	177 000	116	100	1
$(NH_4)_2SO_4$ precipitate	131.8	49 000	373	28	3.2
Sephadex G-100 eluate	10.0	52 600	5 260	30	46
Sephadex G-25 eluate	1.7	40 300	23 750	23	204

* One unit of inhibitor is the amount which caused 1% inhibition of chitin synthetase.

appeared at or near the void volume. The nature of this material and its relationship to the bulk of the inhibitor have not been determined.

Properties of the inhibitor

The inhibitor present in the crude extract was highly unstable and was completely destroyed after 1 day at 0°C. Presumably, the proteases present in the crude extract destroyed the inhibitor. After partial purification, the inhibitor became much more stable; e.g., after precipitation with $(NH_4)_2SO_4$, the preparation lost only 25% of its inhibitory activity after 3 days at 0°C. The purified inhibitor was only slowly destroyed upon storage at 0°C in the presence of 0.02% NaN_3 for several weeks but freezing quickly destroyed most of the inhibitory activity of the purified preparation.

The inhibitor was digested by proteases. Trypsin (0.8 mg/ml) completely destroyed the activity of the inhibitor (0.1 mg/ml) in 3 h. Acid protease from *Rhizopus chinensis* (0.16 mg/ml) destroyed 70% of inhibitor (0.1 mg/ml) in 3 h.

The molecular weight of the inhibitor was estimated by gel filtration in columns of Sephadex G-100 and Bio-Gel P-60. When the columns were eluted with phosphate/magnesium buffer, the elution volume of the inhibitor corresponded to a molecular weight of 10 000. However, because of the observed retardation of inhibitor in Sephadex columns (see above), the gel filtration was repeated with 0.5 or 1.0 M NaCl in the eluting buffer. At either NaCl concentration, the elution volume of the inhibitor in Bio-Gel P-60 indicated a molecular weight of 17 500. This value is probably a better estimate of the molecular size of the inhibitor.

When the purified inhibitor was heated at 50, 60, 70 or 80°C, there was only a partial loss of inhibitory activity (41—44%). These results are in contrast with the complete destruction of the inhibitor observed in the supernatant fraction heated at 70°C. Presumably, heating accelerated the proteolytic destruction of the crude inhibitor, or the inhibitor was lost by co-precipitation with proteins coagulated during heating. A similar case was reported for the Y inhibitor of baker's yeast carboxypeptidase which is thermolabile in crude extracts but thermostable in purified form [14].

The inhibitor was effective against crude (mixed membrane fraction) or puri-

fied (chitosome) preparations of chitin synthetase of *M. rouxii* (Fig. 3). Since both preparations contained a large proportion of chitin synthetase in a zymogenic state, proteases were routinely added to the assays to activate the zymogen. The inhibitor was effective regardless of the protease (acid protease from *Rhizopus chinensis*, Rennilase, trypsin or mexicain) used to activate the zymogen.

The inhibitor had no effect on the digestion of casein or haemoglobin by trypsin, acid protease from *Rhizopus chinensis*, mexicain, papain or Rennilase. Also, it did not inhibit the endogenous proteolytic activity of the mixed membrane fraction from yeast of mycelial cells of *M. rouxii*, measured with either casein (pH 6.0), haemoglobin (pH 3.0), or fibrin blue (pH 2.5) and it had no inhibitory effect of Azocoll hydrolysis by a crude neutral protease from *Phycomyces blakesleeanus* known to activate the chitin synthetase of this fungus (Fischer, P., personal communication). Finally, the purified inhibitor showed no proteolytic activity against casein (pH 6.0) or haemoglobin (pH 3.0).

Further evidence that the inhibitor of *M. rouxii* did not prevent activation of the chitin synthetase zymogen but blocked directly the operation of active chitin synthetase was obtained in the following experiments. Purified chitosomes, from yeast cells of *M. rouxii*, were preactivated with either trypsin or Rennilase in the presence and absence of the inhibitor. Proteolysis was stopped by addition of soybean trypsin inhibitor or pepstatin A respectively. Inhibitors and proteases were then removed from the chitosomes by filtration through a Bio-Gel A-5m column. The activated chitosomes, which eluted in the void volume, were assayed for chitin synthetase activity (no protease was added to assay mixtures). As shown in Table II, the inhibitor did not affect the activation of

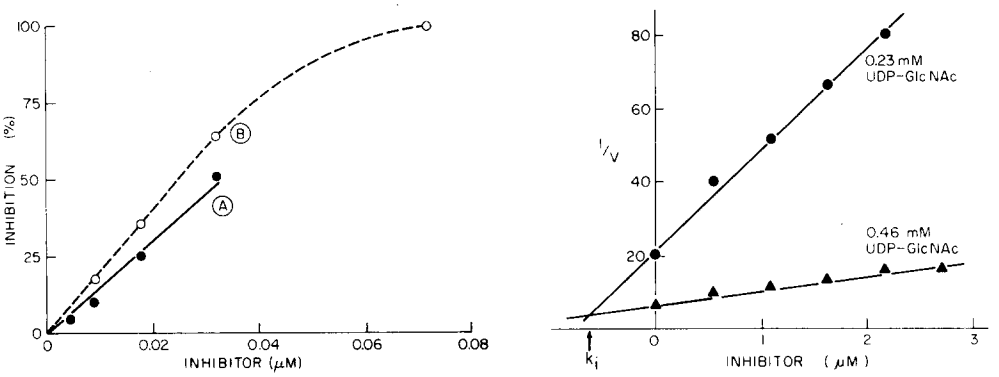

Fig. 3. Effect of inhibitor on chitin synthetase. A. Brij-treated mixed membrane fraction (4 mg protein) was incubated with Rennilase (3.33 mg/ml) & with several concentrations of purified inhibitor in a final volume of 0.125 ml. All other reagents were in the usual concentration in the chitin synthetase assay. After 30 min, the reaction was stopped with 1 M NaOH and the whole mixture filtered, washed once with 1 M NaOH & twice with approx. 20 ml of 1 M acetic acid/95% ethanol (80 : 20, v/v). The filters were dried, placed in scintillation vials with 3 ml scintillation fluid and counted. B. Mycelial chitosomes (17.5 μg protein) were incubated with Rennilase (800 μg/ml) at various concentrations of inhibitor. After 1 h the reaction was stopped, and the mixture treated as in A. Inhibition was calculated as described in Methods.

Fig. 4. Kinetics of inhibition of chitin synthetase. Samples of Brij-treated mixed membrane fraction were incubated with trypsin (0.4 mg/ml) and various concentrations of inhibitor at two substrate concentrations. Data were plotted according to the method of Dixon [15].

TABLE II

EFFECT OF CHITIN SYNTHETASE INHIBITOR ON THE PROTEOLYTIC ACTIVATION OF CHITIN SYNTHETASE

Two samples of chitosomes were pre-incubated with 25 μg trypsin (Expt. A) or 5 mg rennilase (Expt. B) either in the absence or in the presence of 1.26 μM inhibitor in a total volume of 2.5 ml (Expt. A) or 1.75 ml (Expt. B). A third sample of chitosomes was incubated with buffer alone. After 30 min at 22°C, the reaction mixtures received 100 μg soybean trypsin inhibitor (Expt. A) or 50 μg pepstatin A (Expt. B) and were subjected to gel filtration through Bio-Gel A-5m. The columns (0.83 × 16.6 cm) were equilibrated and eluted with phosphate/magnesium buffer containing the corresponding protease inhibitor. The chitosomes were recovered in the void volume, and their chitin synthetase activity was measured without addition of protease and expressed in units per mg protein.

Pre-incubation conditions	Chitin synthetase activity (units)	
	Experiment A (trypsin)	Experiment B (Rennilase)
Buffer	0.06	0.04
Protease	0.47	0.94
Protease plus inhibitor	0.51	1.00

chitin synthetase zymogen by trypsin or Rennilase. In a similar experiment, chitosomes were preactivated in the same manner with either trypsin or Rennilase; proteolysis was stopped by soybean trypsin inhibitor or pepstatin A, respectively. Protease and inhibitor were removed and the activated chitosomal enzyme was assayed in the presence and absence of inhibitor. In either case, the inhibitor caused a 35% reduction in enzymatic activity (Table III). In a further experiment, purified chitosomes from yeast cells of *M. rouxii* were preactivated with Rennilase (800 μg/ml) and proteolysis stopped by addition of pepstatin A (10 μg/ml). The chitosomes were then added to chitin synthetase assay mixtures in the presence and absence of 2.5 μM inhibitor. Samples were taken at intervals of 10 min for 1 h. In both cases, the reaction proceeded at a linear rate but, in the presence of inhibitor there was a 40% reduction in chitin synthetase activity.

Kinetic analysis of inhibition of activity of chitin synthetase showed that the inhibitor competed with the substrate, UDP-GlcNAc (Fig. 4). The K_i value for the inhibitor, calculated as described by Dixon [15], was 0.63 μM.

TABLE III

EFFECT OF INHIBITOR ON CHITIN SYNTHETASE ACTIVITY OF PREACTIVATED CHITOSOMES

Chitosomes were preactivated and treated as described in Table II. The chitin synthetase activity of the chitosomes was then assayed in the absence or presence of 1.26 μM inhibitor.

Chitosomes preactivated with	Chitin synthetase activity (units)		% Inhibition
	without inhibitor	with inhibitor	
Trypsin	0.307	0.200	35.0
Rennilase	0.630	0.415	34.0

Discussion

Contrary to what had been previously suspected [6,7,9], the protein inhibitor of chitin synthesis from *M. rouxii* turned out to be quite different from the one isolated from *Saccharomyces cerevisiae* by Ulane and Cabib [5]. Besides a two-fold difference in molecular weight, the two inhibitors differ sharply in modus operandi. The inhibitor from *S. cerevisiae* (mol. wt. approx. 8500) acts indirectly by inhibiting the endogenous protease (proteinase B) [2,3] that activates the chitin synthetase zymogen. In contrast, the inhibitor from *M. rouxii*, presently described (mol. wt. approx. 17 500), does not inhibit proteases but blocks directly the operation of active chitin synthetase. Since proteinase B affects various other enzymes [16], the inhibitor isolated from *Saccharomyces* [5] cannot be considered specific for chitin synthesis. On the other hand, since the inhibitor from *M. rouxii* competes with UDP-GlcNAc, it is probably specific for chitin synthetase. Our data do not rule out the possibility that *M. rouxii* may also contain an inhibitor(s) of the activating protease(s) of chitin synthetase zymogen, similar to that found in *Saccharomyces* [5]. The role of the chitin synthetase inhibitor of *M. rouxii* in vivo has yet to be established; presumably, it is an additional component of the regulatory mechanism of chitin synthesis.

Acknowledgements

This work was supported in part by a research grant, AI-05540, from the National Institutes of Health, Bethesda, Md., U.S.A., and grants 071 and 847, from the Consejo Nacional de Ciencia y Technología, México.

References

1 Cabib, E. and Farkas, V. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2052—2056
2 Cabib, E. and Ulane, R. (1973) Biochem. Biophys. Res. Commun. 50, 186—191
3 Ulane, R.E. and Cabib, E. (1976) J. Biol. Chem. 251, 3367—3374
4 Cabib, E. and Keller, F.S. (1971) J. Biol. Chem. 246, 167—173
5 Ulane, R.E. and Cabib, E. (1974) J. Biol. Chem. 249, 3418—3422
6 McMurrough, I. and Bartnicki-Garcia, S. (1973) Arch. Biochem. Biophys. 158, 812—816
7 Ruiz-Herrera, J. and Bartnicki-Garcia, S. (1976) J. Gen. Microbiol. 97, 241—249
8 Bracker, C.E., Ruiz-Herrera, J. and Bartnicki-Garcia, S. (1976) Proc. Natl. Acad. Sci. U.S. 73, 4570—4574
9 Ruiz-Herrera, J., Lopez-Romero, E. and Bartnicki-Garcia, S. (1977) J. Biol. Chem. 252, 3338—3343
10 Schlamowitz, M. and Peterson, L.U. (1959) J. Biol. Chem. 234, 3137—3145
11 McDonald, C.E. and Chen, L.L. (1965) Anal. Biochem. 10, 175—177
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Castañeda-Agulló, M., Hernandez, A., Loaeza, F. and Salazar, W. (1945) J. Biol. Chem. 159, 751
14 Matern, H., Hoffmann, M. and Holzer, H. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4874—4878
15 Dixon, M. (1953) Biochem. J. 55, 170—171
16 Holzer, H., Betz, H. and Ebner, E. (1975) in Current Topics in Cell Regulation (Horecker, B.L. and Stadtman, R., eds.), Vol. 9, pp. 103—156, Academic Press, New York