Rapid turnover of tryptophan hydroxylase in serotonin producing cells: demonstration of ATP-dependent proteolytic degradation

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Abstract A rapid and continuous proteolysis of tryptophan hydroxylase was demonstrated with two mast cell lines derived from rat basophilic leukemia cells (RBL2H3) and mouse mastocytoma (FMA3). Under conditions in which protein biosynthesis was arrested by administration of cycloheximide, the decay profile of tryptophan hydroxylase protein was traced by Western blot analysis. Incorporation of [^{35}S]methionine and the chase experiment performed without interfering with the metabolic stage also showed that tryptophan hydroxylase had been cleaved rapidly. The half life of the enzyme was 11–15 min in RBL2H3 cells and 40–60 min in FMA3 cells, and the process was demonstrated to be dependent on intracellular ATP.

Key words: Tryptophan hydroxylase; Serotonin biosynthesis; Mast cell; Proteolysis

1. Introduction

The largest reservoirs of serotonin in the mammalian body are the blood circulation and the gastrointestinal mucosa [1,2]. Gastrointestinal enterochromaffin-cells and mast cells of rodents are serotonin producing whereas platelets are not. Most body serotonin in these animals is produced by these non-neural cells. Tryptophan hydroxylase is the rate limiting enzyme of serotonin biosynthesis. The non-neural form of the enzyme has been described in gastrointestinal mucosa [3,4] and has also been studied in cultured cells of mouse mastocytoma origin [5–10]. The non-neural enzyme is a different entity from the neural enzyme. However, the molecular basis of the difference between the two types has not been understood even after considerable investigation [7,8,11–13]. An immunohistochemical study revealed that distribution of serotonin and tryptophan hydroxylase in murine gastrointestinal mucosa did not always parallel quantity [14]. Such heterogeneity of distribution suggests that the serotonin producing pathway is attenuated in relation to cell age, tissue specific localization, or to adaptation to physiological conditions. Little is known concerning the regulation of peripheral serotonin production. Actually, RBL2H3 cells show an increase in tryptophan hydroxylase activity as high as 10-fold within a short period after stimulations (to be published elsewhere). The aim here was to find a possible mechanism which could afford a rapid restoration of storage serotonin after a vigorous discharge evoked by immune stimulation. A rapid and continuous proteolysis of tryptophan hydroxylase was demonstrated in this study. This degradation was shown to mask rapid biosynthesis under an apparent low steady state of enzyme activity in living but presumably resting cells.

2. Materials and Methods

Cycloheximide was purchased from Sigma (St.Louis, MO). The [^{35}S]methionine and [^{35}S]cysteine mixture (Tran^{35}S-label) was purchased from ICN (Costa Mesa, CA). Dulbecco's Modified Eagle's Medium (DMEM), RPMI1640, and its methionine-free derivatives were purchased from Gibco BRL (Gaithersburg, MD). Rabbit antiserum was raised against purified tryptophan hydroxylase of mouse mastocytoma, P815 [15]. RBL2H3, a mast cell line derived from basophilic leukemia cells, was obtained from The Japanese Cancer Research Resources Bank (Tokyo). RBL2H3 cells were kept as a monolayer culture in DMEM containing 10% fetal calf serum and under 5% CO_2/95% air. FMA3 cells of a mouse mastocytoma cell line were maintained as a suspension culture in RPMI1640 containing 10% fetal calf serum. Five hours before analyses, cells were placed in serum-free media buffered with hepes/NaOH instead of with $NaHCO_3/CO_2$ alone.

Hydroxylation of tryptophan in living cells under monolayer culture was estimated by measuring accumulation of 5-hydroxy-L-tryptophan (5HTP) after administration of a high dose of NSD-1015, an inhibitor of aromatic L-amino acid decarboxylase. Cells were placed in fresh culture medium containing 400 μM NDS-1015 and kept at 37°C for 30 min. The culture was terminated by addition of 5% ascorbic acid in 1 M HCl. The whole culture including the medium was subjected to analysis of accumulated 5HTP.

Cell-free enzyme activity was determined essentially as described [15] with the modifications mentioned below. Culture medium was removed by suction (in the case of a monolayer culture of RBL2H3) or by centrifugation (FMA3). Cells were placed in 20 μl of phosphate buffered saline then subjected twice to freezing in liquid nitrogen and thawing in running tap water. The disrupted cells were incubated in the mixture consisted of 30 mM DTT, 50 μM Fe^{2+}, 4 mg/ml catalase in 0.1 M Tris-acetate (pH 8.1) for 10 min at 30°C. Then, another cocktail was added to complete the reaction mixture, resulting in a final concentration of tryptophan; 250 μM, 6*R*-tetrahydrobiopterin; 400 μM, NADH; 500 μM and NSD-1015 1 mM, catalase; 2 mg/ml and 4.5 μg/ml of sheep liver dihydropteridine reductase (Sigma). The reaction was allowed to proceed for 10 min at 30°C. The 5HTP formed was measured using high performance liquid chromatography.

Methionine incorporation was performed as follows. The cells were incubated at 37°C for 60 min in methionine-free medium buffered with hepes/NaOH containing 10% dialyzed fetal calf serum. The cells were then exposed to [^{35}S]methionine (7.4 MBq/ml, 41.88 TBq/mmol) dissolved in methionine-free medium. For the chase experiments, the radioactive medium was replaced with non-radioactive medium enriched with 5 × methionine and the culture continued. Since radioactive cysteine was diluted with the non-radioactive amino acid in the methionine-free medium, we neglected to consider the possible contribution of the labelling of cysteine residues. The culture was terminated and solubilized by addition of 1% NP-40 in 50 mM Tris-HCl (pH 7.8), then immunoprecipitation was performed using rabbit polyclonal anti-tryptophan hydroxylase serum as the primary antibody and Staphylococcal ghosts (Pansolbin, Calbiochem, La Jolla, CA) as a precipitant. The precipitates were subjected to SDS-polyacrylamide electrophoresis followed by fluorographic analysis.

Western blot analysis: after SDS-polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose filter (Advantec, Tokyo)

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by electrophoresis across the gel [16]. The proteins were visualized using rabbit polyclonal anti-tryptophan hydroxylase serum as the primary antibody and horse radish peroxidase-conjugated goat antibody to rabbit IgG (Wako, Tokyo) as the secondary antibody.

3. Results

3.1. Disappearance of tryptophan hydroxylase after cycloheximide treatment

The accumulation rate of 5HTP after administration of a sufficient amount of NSD-1015 was about 0.4–0.7 pmol/min/10^6 cells under culture. The intracellular activity of tryptophan hydroxylase was very sensitive to cycloheximide. Administration of this reagent at concentrations as low as 0.1 μg/ml virtually abolished intracellular hydroxylation of tryptophan to 5HTP within 4 h (Fig. 1). We examined whether the slowdown of the intracellular process was due to the disappearance of tryptophan hydroxylase or to other factors. Enzyme activity was therefore measured in a cell-free assay system. The cell-free enzyme activity of RBL2H3 cells was 4.68 ± 1.76 pmol/min/10^6 cells (n = 24). A rapid disappearance of tryptophan hydroxylase activity was observed after administration of cycloheximide (10 μg/ml) to these cells (Fig. 2a). Cycloheximide concentrations higher than 10 μg/ml did not appear to make the decrease more rapid in the tryptophan hydroxylase activity. Western blot analysis of cell extracts prepared after cells were treated for a short-period with cycloheximide demonstrated a corresponding decrease in the amount of tryptophan hydroxylase protein of which the reported molecular weight is 53,000 Daltons [17] (Fig. 2b). The enzyme activity after 30 min of exposure to cycloheximide (10 μg/ml) was less than 50% of the initial level (the half life was roughly 11 min based on data of the first 60 min). After washing cycloheximide out of the culture medium, about 50% of the initial enzyme level was restored within 1 h, indicating that cellular functionality remained intact (data not shown). With FMA3 cells, the observed 50%-decay time was 40–60 min and the initial level of tryptophan hydroxylase activity was 100–150 pmol/min/10^6 cells, roughly 30-fold higher than with RBL2H3 cells.

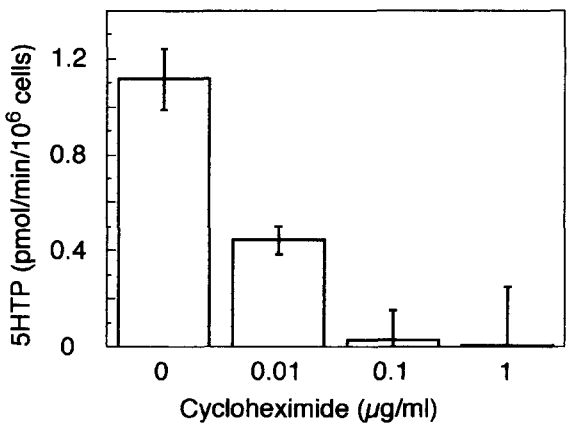

Fig. 1. Inhibition of protein biosynthesis abolishes endogenous conversion of tryptophan to 5HTP in RBL2H3 cells. RBL2H3 cells (average density: 1.5×10^5 cells per well of a 96-well microassay plate) were placed in serum-free DMEM buffered with hepes-NaOH 1 h prior to the experiment, then treated with the indicated concentrations of cycloheximide for 4 h. The culture medium of each well was then replaced with fresh media and 400 μM NSD-1015 were added for 5HTP accumulation, as described in section 2 (n = 6 for each point).

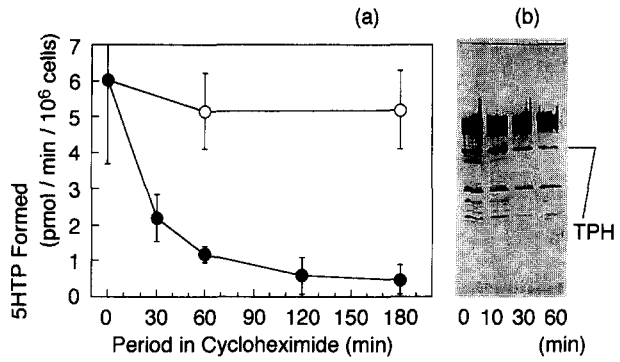

Fig. 2. Effect of arresting protein biosynthesis by cycloheximide. RBL2H3 cells were exposed to cycloheximide (1 μg/ml) for the indicated periods. (a) Tryptophan hydroxylase activity was measured with disrupted cells, as described in section 2. (b) After the indicated times of cycloheximide administration, cells were sonicated. Extracts were then prepared by centrifugation at 12,000 rpm for 10 min and subjected to SDS-polyacrylamide gel electrophoresis for Western blot analysis. TPH: tryptophan hydroxylase.

3.2. Pulse chase of tryptophan hydroxylase labeled with [^{35}S]methionine

The question remained as to whether the observed rapid degradation was due to an artifact evoked by administration of cycloheximide. In order to clarify this point, turnover was examined without administering cycloheximide. Tryptophan hydroxylase was labeled in situ by incorporation of [^{35}S]methionine, then immunoreactive protein bands were chased using specific antiserum. On labeling, only a faint band was recognized corresponding to the intact subunit of tryptophan hydroxylase (Mr = 53,000), whereas an extremely dense band with an Mr < 20,000 appeared early and continued to increase in density (Fig. 3a). In the chase experiment (Fig. 3b), the presumably intact subunit faded with time, corresponding to the observed decay time of enzyme activity. In the similar experiments using FMA3 cells, an even more faint band with an Mr = 53,000 appeared (data not shown). These results indicate that newly synthesized tryptophan hydroxylase was nearly simultaneously degraded.

3.3. Dependence on ATP

In order to observe the dependence of this process on ATP, metabolic inhibitors were applied to the cell to deplete ATP within the cell. RBL2H3 cells were placed in 2,4-dinitrophenol (0.5 mM) and 2-deoxyglucose (30 mM) in the absence of cycloheximide, however, tryptophan hydroxylase activity stayed at the initial level for about 1–2 min then dropped as if protein biosynthesis were blocked, leaving the degradation process unchanged. Simultaneous administration of these reagents with cycloheximide did not yield significantly different results from those using cycloheximide alone. With FMA3 cells of the mouse mastocytoma cell line, the effect of cycloheximide was effectively antagonized by the addition of the two reagents as shown in Fig. 4. Either 2,4-dinitrophenol (0.5 mM) alone or 2-deoxyglucose (30 mM) did not completely remove the effect

of cycloheximide. These reagents did not raise the level of tryptophan hydroxylase in the absence of cycloheximide.

4. Discussion

We previously demonstrated that a large proportion of tryptophan hydroxylase is in quiescent form in specific regions [3,4,10]. The major limiting factor preventing the enzyme from being fully active is most probably an insufficient supply of intracellular ferrous iron [18]. The actual activity of tryptophan hydroxylase in RBL2H3 cells was only one tenth of the potential activity of the enzyme under our culture conditions used. Furthermore, the intracellular hydroxylation of tryptophan was very sensitive to cycloheximide, suggesting that the enzyme system might depend on the 'fragile' component. The possibility that the 'fragile' component was tryptophan hydroxylase itself was supported by the following: (1) enzyme activity measured in the cell-free system also decreased after cycloheximide administration, (2) concomitant protein degradation was visualized by Western blot analysis using specific antiserum, and (3) rapid turnover was observed without using cycloheximide by means of a radioisotope tracer technique. The rapid disappearance of tryptophan hydroxylase by cycloheximide treatment was demonstrated to be closely dependent on ATP production in the cell (Fig. 4), suggesting that the proteolysis was an ATP- dependent process in the cell. Since the biosynthesis of tryptophan hydroxylase and its degradation seem to be counter-balanced under ordinary conditions, we expected that temporary depletion of ATP would lead to a transient rise in the amount of enzyme, if the decrease in the biosynthesis would commence after any delay. However, we could not observe this rise in both lines of cells. In the case of RBL2H3 cells, in which tryptophan hydroxylase disappears faster than in FMA3 cells, the enzyme did not maintain its level in the presence of 2,4-dinitrophenol (0.5 mM) and 2-deoxyglucose (30 mM) even in the absence of cycloheximide (data not shown). This indicates that the protein biosynthesis of tryptophan hydroxylase in RBL2H3 cells was even more sensitive to ATP depletion. The

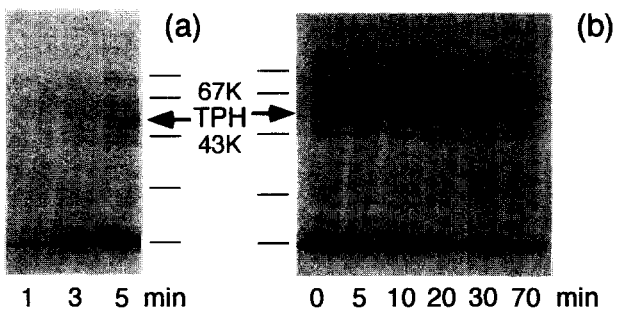

Fig. 3. Incorporation of [^{35}S]methionine into tryptophan hydroxylase and disappearance of the major peptide. RBL2H3 cells were fed with [^{35}S]methionine for 5 min (left). The cells were then washed and kept in non-radioactive culture medium (right). Proteins collected by immunoprecipitation by using polyclonal anti-tryptophan hydroxylase rabbit serum were subjected to SDS-polyacrylamide gel electrophoresis. A protein band correspondent to tryptophan hydroxylase (indicated as TPH, Mr = 53,000) was very faint. A protein band correspondent to tryptophan hydroxylase (indicated as TPH, Mr = 53,000) was very faint. A very dense band, close to the dye front, appeared and increased in the early incubation (right), then decreased in the chase (left). Thin lines represent marker proteins, from top to bottom; 94k, 67k, 43k, 30k, and 21.1k (14.4k was not separated from 21.1k).

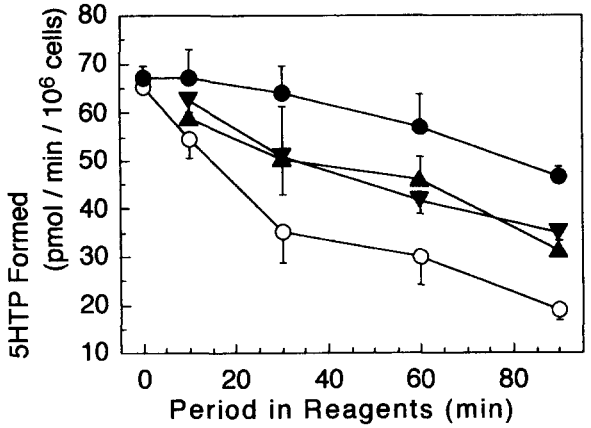

Fig. 4. Dependence of tryptophan hydroxylase turnover on intracellular ATP. At zero-time, FMA3 cells (3×10^5 cells/ml) were placed in 10 μg/ml of cycloheximide alone (○), or in the presence of 2,4-dinitrophenol (▲, 500 μM) or 2-deoxyglucose (▼, 30 mM), or both 2,4-dinitrophenol and 2-deoxyglucose (●).

measured half life of tryptophan hydroxylase in the typical cases depicted in Figs. 2 and 3 was about 10 to 15 min. However, the half life of tryptophan hydroxylase in this cell line varied widely between 10 to more than 60 min in RBL3H3 cells. It was also noted that steady state enzyme activity varied widely, as mentioned above. We speculated that the steady state level may be primarily determined by degradation at a constant rate of biosynthesis. If this were the case, working out the equation for equilibrium dynamics would give a solution indicating that the steady state level is proportional to the half life. However, we could find no positive correlation between the half life and the steady state level of tryptophan hydroxylase (correlation coefficient, $r = -0.47$ with 18 experiments), indicating that the rate of biosynthesis was not constant in each cells of the different culture. Since the biosynthetic rate must be equal to the degradation rate over the short term, cells with a high rate of enzyme production would have an even higher rate of enzyme degradation. We are not able to offer a reasonable explanation for these observations as yet. Nevertheless, rapid proteolysis is obviously a major influence in determining the content of tryptophan hydroxylase in the cell. The rate of degradation may be sensitive to the cell's physiological conditions.

The half life of tryptophan hydroxylase in the rat central nervous system and in the spinal cord was reported to be 2–3 days [19] and the decay rate in rat raphe dorsalis was found to be 0.3–0.4 days^{-1} [20]. These rates are too slow to be compatible with a rapid regulatory response. Dumas et al. reported that the mRNA level of tryptophan hydroxylase was much higher in pineal gland than in the brain stem. They concluded that translation was regulated differently in these tissues but they did not report on protein turnover. Sitaram and others examined the well-known instability of pineal tryptophan hydroxylase and found that the enzyme's half life was 75 min as measured in rats following administration of cycloheximide [21]. Rapid inactivation of the enzyme may have resulted in degradation via non-specific cellular scavenging mechanisms. Our results suggest that rapid turnover is driven by specific proteolysis targeting tryptophan hydroxylase rather than by a mopping-up mechanism for scavenging unwanted proteins. The mechanism by

which the proteolytic process is boosted by ATP remains to be elucidated.

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