Reticulocyte lipoxygenase, ingensin, and ATP-dependent proteolysis

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Lipoxygenase purified from rabbit reticulocyte lysate has a molecular mass of 68 kDa on SDS gel and a p*I* of 5.97. Lipoxygenase is inhibited by nordihydroguaiaretic acid (NDGA), 3-amino-1-(*m*-(trifluoromethyl)phenyl)-2-pyrazoline (BW755C), 5,8,11,14-eicosatetraynoic acid (ETYA), salicylhydroxamate (SHAM) or hemin. Metal ions or nucleotides do not affect its activity. The addition of certain of these inhibitors to the reticulocyte extract also inhibited the ATP-dependent proteolysis of casein, one of the distinct characteristics of reticulocytes. No clear correlation between lipoxygenase activity and ATP-dependent proteolysis could be detected. Hemin and NDGA inhibited both processes, but the concentrations necessary for inhibition were quite different. SHAM completely inhibited lipoxygenase, but not proteolysis. *o*-Phenanthroline inhibited ATP-dependent proteolysis, but had no effect on lipoxygenase activity. We have also purified a high-molecular-mass protease, ingensin, from reticulocyte extract. This protease accounted for more than 90% of the casein-degrading activity in reticulocyte extract. NDGA inhibited ingensin at the same concentrations required for inhibition of ATP-dependent proteolysis. These results suggest that lipoxygenase is not indispensable for the ATP-dependent proteolysis and the novel high-molecular-mass protease, ingensin, may be involved in the process.

Reticulocyte *Lipoxygenase* *Protease* *ATP-dependent proteolysis* *(Rabbit)*

1. INTRODUCTION

Lipoxygenase is an enzyme which oxygenates unsaturated fatty acids such as arachidonic acid or linoleic acid. The 3 distinct enzymes in mammalian tissues are classified as 5-, 12- and 15-lipoxygenases according to the specific site of oxygenation. This activity is low in most tissues, but reticulocytes contain about 1000-fold more activity than other cells. However, its biological role in reticulocytes remains unknown [1]. Rapoport and co-workers [2–8] have described several enzymatic properties of reticulocyte lipoxygenase and have suggested that it is a 15-lipoxygenase involved in the ATP-dependent degradation of mitochondrial proteins and pulse-labeled cytosolic proteins. It is limited to these cells, catalyzing 1–2% of intracellular protein breakdown. These findings have not been confirmed by other investigators and controversies remain concerning the involvement of lipoxygenase in the energy-dependent breakdown of proteins. Other possible mechanisms of ATP-dependent proteolysis have been postulated, such as ATP-dependent conjugation of ubiquitin to substrate proteins or ATP-dependent protease(s) (review [9]).

This paper reports the enzymatic characteristics of purified lipoxygenase and discusses the possibility that it is involved in ATP-dependent proteolysis in reticulocyte extracts.

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2. MATERIALS AND METHODS

2.1. *Assay of lipoxygenase*

Lipoxygenase was determined spectrophotometrically according to Rapoport et al. [1]. Typically, 1 ml reaction mixture contained 50 mM phosphate buffer, pH 7.4, and 0.1 mM linoleic acid. The formation of fatty acid hydroperoxide was assayed by the absorption at 234 nm. In some experiments lipoxygenase was assayed using [^{14}C]arachidonic acid as a substrate [10] and measuring the decrease of arachidonic acid. The radioactive metabolites were separated by thin-layer chromatography and counted.

2.2. *Purification of lipoxygenase from rabbit reticulocytes*

Reticulocytes were produced by successive injections of phenylhydrazine [11,12]. Reticulocytes (40 ml) were lysed by the addition of 1.2 vols of 1 mM dithiothreitol and ultrasonication, and centrifuged at 30000 × *g* for 1 h. To the resulting supernatant solid ammonium sulfate was added to a final concentration of 60%. The precipitate was dissolved in 3 ml distilled water and dialyzed against 5 mM phosphate buffer, pH 7.5. The dialyzed crude extract was loaded on a DEAE-cellulose column (1 × 20 cm) equilibrated with 5 mM phosphate buffer, pH 7.0. After extensive washing with the same buffer (until the red color of hemoglobin disappeared), a linear gradient of 0–400 mM NaCl in 200 ml phosphate buffer was run. Lipoxygenase activity was then eluted at 20–30 mM NaCl. Fractions containing lipoxygenase activity were then loaded on a hydroxyapatite column (1 × 10 cm) equilibrated with 5 mM phosphate buffer, pH 7.4. The activity was eluted with a 140 ml linear gradient of 5–300 mM phosphate. Those fractions containing lipoxygenase activity were pooled and stored at 4°C for up to 7 days. Lipoxygenase was further purified by preparative isoelectric focusing in Ampholine, pH 5–8 (fig.1)

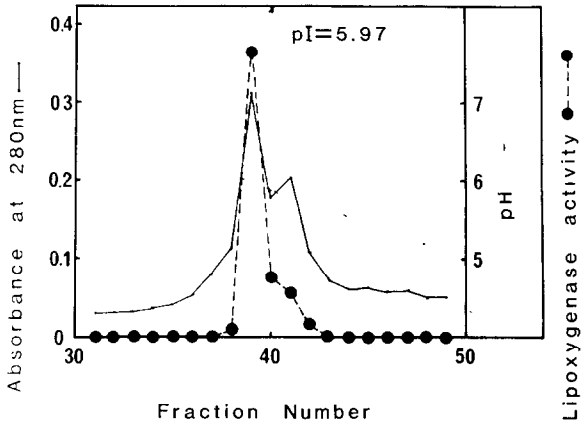

Fig.1. Preparative isoelectric focusing of reticulocyte lipoxygenase. The fractions obtained by hydroxyapatite that contained lipoxygenase activity were pooled and loaded on an isoelectric focusing column (LKB) using Ampholine as a carrier. The activity of lipoxygenase was assayed as described in section 2.

2.3. *Assay of proteolytic activity*

The substrate for proteolytic assays was prepared by reductive methylation of casein (kindly provided by Dr K. Hanada, Taisho Pharm. Co., Ohmiya). Caseinolytic activity was determined by the release of ^{14}C-labeled peptides soluble in trichloroacetic acid. The reaction mixture contained 400 μg [^{14}C]casein in 100 μl of 50 mM Tris-HCl, pH 8.0. The amount of casein in the assay mixture should be no less than 400 μg because ATP dependency tends to vary at the low substrate concentrations which have been typically employed by other investigators [11–13]. The assay mixture was incubated at 37°C for 60 min, and the reaction terminated by the addition of 400 μl of 10% trichloroacetic acid. After 1 h on ice, the solution was centrifuged, and 200 μl of the supernatant was added to 4 ml nonion scintillator.

The casein-degrading activity generally accompanies succinyl-leucyl-leucyl-valyl-tyrosine-methyl-coumarinamide (SLLVT)-degrading activity. Therefore we measured this activity upon purification. The reaction mixture contained 0.3 mM SLLVT in 20 mM Tris-HCl, pH 9.0 [14].

2.4. *Purification of the high-molecular-mass protease, ingensin, from rabbit reticulocytes*

Reticulocyte extract was prepared as described above. On DEAE-cellulose the protease activity was eluted at 250 mM NaCl. The active fractions were collected, concentrated with ammonium sulfate (60% saturation) and dialyzed with 5 mM phosphate buffer, pH 7.0. The crude protease fraction was separated by gel filtration with an HPLC TSK3000SWG column in 0.1 M phosphate

buffer, pH 7.0. The active fraction was dialyzed with distilled water and loaded on a hydroxyapatite column (1 × 10 cm) equilibrated with 5 mM phosphate buffer, pH 7.0. The activity was eluted with a 140 ml linear gradient of 5–300 mM phosphate. The enzyme was eluted at a molecular mass of 700–1000 kDa by HPLC. Gel electrophoresis without SDS showed a single band, but SDS gel electrophoresis revealed several bands in the range of 25–35 kDa. The results were reproducible from preparation to preparation, and from tissue to tissue [14–16]. The protease appears to consist of about 20 subunits and was named ingensin [14].

3. RESULTS

3.1. *Characterization of reticulocyte lipoxygenase*

The SDS gel electrophoresis profiles of the polypeptide present in the purified lipoxygenase preparation are shown in fig.2a. The enzyme was highly purified to homogeneity as shown in fig.2. A polypeptide of 68 kDa co-migrated with enzyme activity during all of the purification steps. This molecular mass agreed with that obtained by gel filtration of crude extract (not shown) on HPLC. The isoelectric point was determined to be 5.97 by isoelectric focusing (fig.1). The two values are slightly different from those reported [1]. Usually, 0.5–2.0 mg lipoxygenase was obtained from 40 ml reticulocytes. The purified lipoxygenase catalyzed the 0.243 unit increase in absorbance at 234 nm/min per μg. The K_m for linoleic acid in the reticulocyte lipoxygenase reactions was 18 μM. The most striking characteristic of the lipoxygenase reaction was its inhibition by substrate. 6 μg of the enzyme underwent 50% inhibition when 80 μM linoleic acid was added and 9 μg enzyme was inhibited by 50 μM substrate.

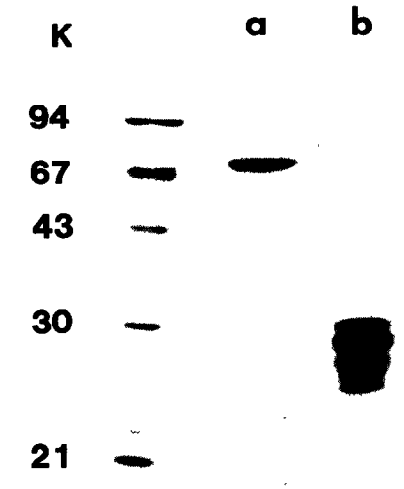

Fig.2. SDS gel electrophoresis of purified reticulocyte lipoxygenase (a) and ingensin (b).

3.2. *Effect of various compounds on lipoxygenase activity*

A number of effectors that have been reported to inhibit other lipoxygenases were tested to determine the effects on the reticulocyte enzyme (table 1). Divalent cations such as Ca^{2+}, an activator of 5-lipoxygenase [10] or Cu^{2+}, an inhibitor of 12-lipoxygenase [17], or chelators had no effect on

Table 1

Effect of various compounds on lipoxygenase activity

Effectors	Final concentration	% activity
No addition		(100)
Metal ions (Ca, Mg, Mn, Ni, Fe, Cu, Hg)	1 mM	100
EDTA, *o*-phenanthroline	1 mM	100
ATP, ADP, AMP	1 mM	100
ETYA	1 μg/ml	0
NDGA	10 μM	0
BW755C	10 μM	21
SHAM	10 μM	0
α-Naphthol	100 μM	72
α-Tocopherol	100 μM	83
Hemin	0.2 μM	47
Protoporphyrin IX	0.2 μM	100
Mepacrin	10 μg/ml	100
Indomethacin	10 μM	100
Cycloheximide	10 μM	83
W7	10 μg/ml	100
Polylysine	100 μg/ml	100

Various reagents were added to the assay mixture, and lipoxygenase activity was assayed under standard conditions. The relative activity is expressed as the percent of activity found in the absence of compounds. Stock solution of the anti-lipoxygenase was initially dissolved in dimethyl sulfoxide (DMSO). DMSO (final concentration 1%) did not inhibit lipoxygenase activity. These data were obtained with purified enzyme (3 μg/assay)

the oxidation of linoleic acid by reticulocyte lipoxygenase. ATP, known as an activator of 5-lipoxygenase of peritoneal polymorphonuclear leucocytes [10], failed to activate either purified reticulocyte lipoxygenase or the enzyme activity in the crude extract. However, several compounds reported to be selective inhibitors of lipoxygenase inhibited the enzyme strongly. Among these, 5,8,11,14-eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA) and salicylhydroxamate (SHAM) inhibited the enzyme completely at concentrations less than 10^{-6} M; BW755C was less effective, but its apparent K_i was 5 μM. The antioxidant α-naphthol or α-tocopherol inhibited lipoxygenase slightly, as reported [3]. Hemin was a potent inhibitor of the lipoxygenase reaction whereas its Fe-depleted analogue protoporphyrin IX did not inhibit (see also fig.3). Mepacrin, an inhibitor of phospholipase A, and indomethacin, an inhibitor of cyclooxygenase, had essentially no effect. Calmodulin is not involved in the lipoxygenase reaction because the specific inhibitor W7 did not affect it. Polylysine did not inhibit lipoxygenase. Cycloheximide slightly inhibited the reaction, but its mechanism of inhibition is unknown. These inhibitors had similar effects on purified lipoxygenase and on the enzyme activity in the crude extract.

3.3. *Possible involvement of lipoxygenase in ATP-dependent proteolysis of exogenous protein in reticulocyte extract*

It has been inferred that lipoxygenase is involved in ATP-dependent protein breakdown in reticulocytes because the ATP-dependent proteolysis of hemoglobin or amino acid analog-containing abnormal proteins was inhibited by the lipoxygenase inhibitor, SHAM [1–8]. To test this possibility, we studied the effect of the inhibitors listed in table 1 on the ATP-dependent breakdown of exogenously added casein.

As shown in table 2, 1 mM SHAM did not inhibit ATP-dependent casein degradation in reticulocyte extract, contrary to the observation of Rapoport et al. [2–8]. Other anti-lipoxygenase such as NDGA and hemin inhibited both ATP-dependent casein hydrolysis of reticulocyte extract and lipoxygenase itself, but the concentrations necessary for inhibition were quite different (fig.3). Hemin inhibited lipoxygenase completely

Table 2

Inhibition of ATP-dependent proteolysis by anti-lipoxygenase reagents

Condition	% activity	
	ATP-independent	ATP-dependent
Reticulocyte extract (R)	(100)	(100)
R + SHAM (100 μM)	100	100
R + SHAM (1 mM)	109	83
R + NDGA (100 μM)	100	75
R + NDGA (1 mM)	100	35
R + BW755C (500 μM)	100	100
R + ETYA (100 μg/ml)	100	100
R + Hemin (10 μM)	93	98
R + Hemin (100 μM)	45	2
R + PPIX (10 μM)	100	91
R + PPIX (100 μM)	94	80
R (anaerobic condition)	100	100

Proteolytic activity of reticulocyte extract was determined as described in section 2. ATP-dependent activity was calculated from the difference between [^{14}C]casein-degrading activities in the presence and absence of added ATP (5 mM). PPIX, protoporphyrin IX. Addition of ATP increased the degradation of casein by 3-fold

at 20 μM, which did not affect proteolysis. NDGA inhibited ATP-dependent proteolysis specifically (apparent K_i = 300 μM), whereas it inhibited lipoxygenase 100% at less than 10^{-6} M. Preliminary observations demonstrated that NDGA inhibited ATP-dependent proteolysis of fatty acid-free reticulocyte extract strongly (fatty acids were removed using a Sep-Pak column), suggesting that fatty acids avoided NDGA-induced inhibition of ATP-dependent proteolysis. ETYA, a specific inhibitor of lipoxygenase, did not affect proteolysis at 100 μg/ml. In contrast, 1 mM *o*-phenanthroline inhibited ATP-dependent proteolysis by 70%, but did not affect lipoxygenase activity (table 1). Moreover, adding lipoxygenase with or without arachidonic acid to reticulocyte extract or to erythrocyte extract which had lost ATP-dependent proteolysis had no effect on proteolysis. Thus no evidence was obtained to demonstrate the involvement of lipoxygenase in ATP-dependent degradation of casein.

We recently purified a fatty-acid activated high-

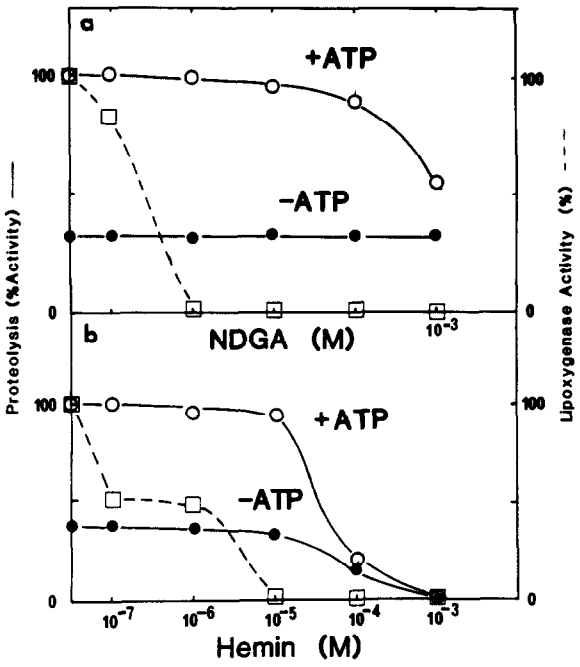

Fig.3. Effects of NDGA or hemin on purified lipoxygenase activity or ATP-dependent proteolysis of reticulocyte extracts. (a) NDGA and (b) hemin were dissolved in DMSO (final concentration, 1%). ATP-dependent (○) or -independent (●) casein-degrading activity of reticulocyte extracts was assayed as described in section 2. Here, the proteolytic activity in the presence of 5 mM ATP was determined as 100%. As shown, NDGA and hemin inhibited purified lipoxygenase (□) at much lower concentrations than those required for inhibition of ATP-dependent proteolysis of casein.

molecular-mass protease, ingensin ([14–16]; see fig.2b), which constitutes about 60% of the [^{14}C]casein-degrading activity in reticulocytes and more than 90% of the activity in erythrocytes at alkaline pH. NDGA inhibited purified reticulocyte ingensin (apparent $K_i = 200\ \mu M$). Therefore a new protease, ingensin, may be involved in the ATP-dependent degradation of exogenously added proteins.

4. DISCUSSION

The molecular properties of the reticulocyte lipoxygenase were different from those reported in [1]. Rapoport has determined the molecular mass as 78 kDa and the *pI* as 5.5. Narumiya et al. [18] have partially purified 15-lipoxygenase from rabbit peritoneal leucocytes and calculated the molecular mass as 61 kDa by gel filtration. The value reported here is intermediate between these. The enzyme is rapidly inactivated by ingensin (S. Ishiura, unpublished) and suitable precautions must be taken during the course of purification. Reticulocyte lipoxygenase is not directly activated by Ca or ATP as 5-lipoxygenase is [10]. Metal ions are not essential for its activity, which distinguishes it from 15-lipoxygenase from peritoneal leucocytes [18]. However, anti-lipoxygenase compounds such as NDGA, ETYA, SHAM and BW755C inhibited reticulocyte lipoxygenase quite strongly.

In mammalian cells, the ATP-dependent proteolysis of endogenous and exogenous proteins was demonstrated only in reticulocyte extracts [11,12] and in liver mitochondria [19]. Attempts to purify ATP-dependent protease(s) in these fractions were unsuccessful; the ATP-dependent casein-degrading activity was reported to disappear after several purification steps. Hershko and co-workers have demonstrated that ATP-dependent proteolysis can be reconstituted from two fractions, one a heat-stable protein called ubiquitin and the other containing three enzymes isolated by affinity chromatography on ubiquitin-Sepharose [20]. They showed that ATP is used in part for conjugation of ubiquitin to substrate protein and ATP is also essential for degradation of the ubiquitin-substrate complex [21]. However, they have failed to demonstrate an ATP-dependent protease. According to Goldberg's and Hershko's group, there is no evidence that reticulocytes, but not erythrocytes, contain distinct proteins (or factors) responsible for energy-dependent breakdown of proteins [9–13].

In 1981, Rapoport demonstrated that ATP-dependent breakdown of pulse-labeled endogenous proteins was inhibited under anaerobic conditions and with the lipoxygenase inhibitor, SHAM [6], suggesting that lipoxygenase was necessary for energy-dependent breakdown of reticulocyte proteins. Moreover, the most suitable substrate of reticulocyte lipoxygenase was isolated mitochondria. From these observations, Rapoport et al. concluded that the main significance of the reticulocyte ATP-dependent proteolysis appeared to be the degradation of mitochondria during maturation of the red cells. This is a very attractive

model because the occurrence of lipoxygenase is highly specific in reticulocytes and the content of the enzyme decreases during maturation [1]. This led us to compare the sensitivities to inhibition of purified lipoxygenase and ATP-dependent proteolysis. We chose casein as the substrate for subsequent experiments for the following reasons. (i) Of several proteins, casein is most susceptible to general proteolysis. (ii) Many exogenous proteins in addition to the pulse-labeled endogenous proteins are degraded by the reticulocyte ATP-dependent proteolytic system, although the ATP dependency differs from substrate to substrate [11,13]. (iii) If ATP is required only for the activity of protease itself or for a marking reaction such as ubiquitin conjugation, there is still a problem in explaining differences in ATP dependency. (iv) There is no report indicating that casein is an inappropriate substrate for studying the ATP-dependent pathway of protein breakdown. (v) If lipoxygenase is involved only in the ATP-dependent degradation of newly synthesized endogenous proteins, but not in that of exogenously added denatured proteins, two distinct mechanisms of ATP dependency should be present. However, no evidence of such mechanisms has been reported.

As shown in fig.3, the sensitivity of both lipoxygenase and ATP-dependent proteolysis to inhibitors was quite different over a wide range of concentrations. Hence there is no evidence indicating that lipoxygenase is involved in ATP-dependent proteolysis. The inhibition of ATP-dependent proteolysis as well as of purified ingensin by high concentrations of NDGA suggests that the newly isolated protease ingensin is involved in ATP-dependent degradation of casein. Rapoport et al. [6] could not observe an inhibition of the ATP-dependent degradation of exogenous substrate BSA by SHAM. We have been unable to demonstrate that SHAM inhibited the degradation of casein. These observations suggest that SHAM does not inhibit the breakdown of all proteins added to reticulocyte extract, indicating that involvement of lipoxygenase in the endogenous proteolysis, if it exists, might be highly selective. We were also unable to demonstrate the inhibition of ATP-dependent breakdown of casein under anaerobic conditions, contrary to the report of Rapoport using pulse-labeled protein. Rapoport and Dubiel [22] also reported that reticulocytes produced by injections of phenylhydrazine differed from those obtained by bleeding. The discrepancy between Rapoport's and our results may be due to qualitative differences of reticulocyte preparations.

In spite of its limited localization and unknown substrate specificity, ATP-dependent proteolysis has been believed to be of importance in living organisms because it is an absolutely cytosolic system. However, recent results suggest that the only general mammalian cytosolic system of proteolysis is Ca-dependent and contains specific proteases; its structure has been elucidated [23–25]. The importance of the Ca-dependent system is apparent in the degradation of stable proteins in disease states or during developmental changes. Quite recently, we have identified a new cytosolic proteolytic system, a fatty acid-dependent proteolysis, in porcine skeletal muscle [14], human placenta [15,16] and rat liver (Yamamoto et al., submitted). However, the physiological significance of this system is unknown at present. The ATP-dependent proteolytic system can be demonstrated under specific conditions with a small amount of exogenous substrate and a 1000-fold excess of endogenous proteins only in reticulocytes. Our results suggest that ATP-dependent proteolysis is a reticulocyte-specific expression of the second cytosolic proteolytic system specifically mediated by a new protease, ingensin. Further study is necessary for elucidating the exceptional ATP-requirement of proteolysis in reticulocyte extract.

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REFERENCES

[1] Rapoport, S., Schewe, T., Wiesner, R., Nalang, K., Ludwig, P., Janicke-Hohne, M., Tannert, C., Hiebsch, C. and Klatt, D. (1979) Eur. J. Biochem. 96, 545–561.

[2] Schewe, T., Halangk, W., Hiebsch, C. and Rapoport, S. (1975) FEBS Lett. 60, 149–152.

[3] Halangk, W., Schewe, T., Hiebsch, C. and Rapoport, S. (1976) Acta Biol. Med. Germ. 36, 405–410.
[4] Muller, M., Dubiel, W., Rathmann, J. and Rapoport, S. (1980) Eur. J. Biochem. 109, 405–410.
[5] Rapoport, S., Dubiel, W. and Muller, M. (1981) Acta Biol. Med. Germ. 40, 1277–1283.
[6] Dubiel, W., Muller, M. and Rapoport, S. (1981) Biochem. Int. 3, 165–171.
[7] Bryant, R.W., Bailey, J.M., Schewe, T. and Rapoport, S. (1982) J. Biol. Chem. 257, 6050–6055.
[8] Kuhn, H., Wiesner, R., Schewe, T. and Rapoport, S. (1983) FEBS Lett. 153, 353–356.
[9] Hershko, A. (1983) Cell 34, 11–12.
[10] Ochi, K., Yoshomoto, T., Yamamoto, S., Taniguchi, K. and Miyamoto, T. (1983) J. Biol. Chem. 258, 5754–5758.
[11] Etlinger, J.D. and Goldberg, A.L. (1977) Proc. Natl. Acad. Sci. USA 74, 54–58.
[12] Etlinger, J.D. and Goldberg, A.L. (1980) J. Biol. Chem. 255, 4563–4568.
[13] Tanaka, K., Waxman, L. and Goldberg, A.L. (1983) J. Cell Biol. 96, 1580–1585.
[14] Ishiura, S., Sano, M., Kamakura, K. and Sugita, H. (1985) FEBS Lett. 189, 119–123.
[15] Ishiura, S., Nojima, M., Yamamoto, T., Fuchiwaki, T., Okuyama, T., Furuya, H. and Sugita, H. (1986) Int. J. Biochem., in press.
[16] Nojima, M., Ishiura, S., Yamamoto, T., Okuyama, T., Furuya, H. and Sugita, H. (1986) J. Biochem. 99, in press.
[17] Nugteren, D.H. (1982) Methods Enzymol. 86, 49–54.
[18] Narumiya, S., Salmon, J.A., Cottee, F.H., Weatherley, B.C. and Flower, R.J. (1981) J. Biol. Chem. 256, 9583–9590.
[19] Desautels, M. and Goldberg, A.L. (1982) J. Biol. Chem. 257, 11673–11679.
[20] Hershko, A., Heller, H., Elias, S. and Ciechanover, A. (1983) J. Biol. Chem. 258, 8206–8214.
[21] Hershko, A., Leshinsky, E., Ganoth, D. and Heller, H. (1984) Proc. Natl. Acad. Sci. USA 81, 1619–1623.
[22] Rapoport, S. and Dubiel, W. (1984) Biomed. Biochim. Acta 43, 23–27.
[23] Ishiura, S. (1981) Life Sci. 29, 1079–1087.
[24] Suzuki, K. and Ishiura, S. (1983) J. Biochem. 93, 1463–1471.
[25] Ohno, S., Emori, Y., Imajoh, S., Kisaragi, M. and Suzuki, K. (1984) Nature 312, 566–570.