Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 364–367, January 1987
Biochemistry

Substrate specificity of the chymotrypsin-like protease in secretory granules isolated from rat mast cells

(mast cell proteases/vasoactive peptides)

HAI LE TRONG, HANS NEURATH, AND RICHARD G. WOODBURY

Department of Biochemistry, SJ-70, University of Washington, Seattle, WA 98195

Contributed by Hans Neurath, September 23, 1986

ABSTRACT **The substrate specificity of rat mast cell protease I (RMCP I), a chymotrypsin-like serine protease localized in the secretory granules of mast cells, was compared to that of bovine α-chymotrypsin by using several peptide and protein substrates of known amino acid sequences. Although the overall specificities of the two proteases appeared similar, subtle but significant differences were observed. RMCP I was more prone than chymotrypsin to hydrolyze peptide bonds consisting of Leu-Xaa or two hydrophobic residues—e.g., Phe-Phe. Additionally, the hydrolysis of angiotensin I catalyzed by chymotrypsin, but not by RMCP I, resulted in the generation of angiotensin II as an intermediate product. In contrast to the solubilized enzyme, the RMCP I activity within the insoluble granules was completely stable for at least 2 months in suitable buffers at pH 8.0 or pH 7.2, at 4°C. Carboxypeptidase A activity associated with isolated mast cell granules was completely inhibited by 10 mM *o*-phenanthroline. Polypeptides smaller than apomyoglobin (17,199 Da) were rapidly hydrolyzed by granule-bound RMCP I, whereas apomyoglobin and other larger proteins were not hydrolyzed. In contrast, the free protease readily hydrolyzed the larger proteins. Neither normal rat serum nor α_1-antitrypsin, both of which inhibited the activity of free RMCP I, was effective in inhibiting granule-associated RMCP I. The results indicate that granule-bound RMCP I is not released into solution from isolated secretory granules under physiological conditions of ionic strength and pH and that the granule structure limits the size of proteins that can be hydrolyzed by the protease.**

Two distinct types of mast cells have been identified in mammalian tissues (1, 2). One type, the mucosal mast cell (MMC), occurs primarily in connective tissue of the mucosa of the respiratory and intestinal tracts. The other cell type, referred to as the connective tissue mast cell (CTMC), is present in loose connective tissue of nearly all nonmucosal tissues and can be isolated as free cells from the peritoneal cavity of rodents.

Mast cells function in inflammation and allergy by secreting granules that contain potent vasoactive agents such as histamine, serotonin, and leukotrienes, as well as high molecular weight proteoglycan (heparin), chemotactic factors, and enzymes. A high proportion of the granule enzymes are serine proteases related to pancreatic chymotrypsin (3, 4). Two chymotrypsin-like rat mast cell proteases have been isolated and characterized—i.e., rat mast cell protease I (RMCP I) from CTMC and rat mast cell protease II (RMCP II) from MMC (5). The functions of the proteases are not known. On the basis of several considerations, however, it is unlikely that the proteases merely catalyze the general degradation of extracellular tissue proteins (6, 7).

Because RMCP I, when secreted into physiological fluids, appears to remain immobilized within the granular matrix (8), we have examined the activities and specificities of granule-associated and free RMCP I, respectively, toward different peptides and proteins of various sizes and known amino acid sequence, some of which may be actual physiological substrates. Additionally, the substrate specificity of RMCP I toward several peptides and proteins was compared to that of bovine chymotrypsin.

MATERIALS AND METHODS

Preparation of Mast Cell Secretory Granules. Peritoneal mast cells, corresponding to CTMC, were isolated from male and female adult Sprague–Dawley rats (Tyler's Laboratory, Bellevue, WA) as described by Lagunoff and Pritzl (9). Typically, the entire peritoneal cell population collected from 50 rats was used to isolate mast cell granules lacking their perigranular membranes (5). For comparative purposes, granules were isolated also from highly enriched (>95%) mast cells (9). The granules were stored at 4°C in 3 mM potassium phosphate buffer, pH 8.0, for several months without loss of RMCP I activity. No RMCP I activity could be detected in the supernatant solution of the granule preparations. Enzymatic activity of the granules, however, was lost upon repeated freezing and thawing.

Purification of RMCP I. RMCP I was purified by affinity chromatography on a column of ovoinhibitor-Sepharose 4B as described previously (5). Preparations of purified RMCP I were analyzed by electrophoresis on polyacrylamide gels (12%) in the presence of sodium dodecyl sulfate and 2-mercaptoethanol (10). The purified protease was stored until needed at −70°C in 50 mM NH_4HCO_3 buffer, pH 7.8, containing 2% (vol/vol) glycerol.

Substrates. Peptide and protein substrates were purchased from Sigma in the highest grade of purity available. Each was examined for purity by reverse-phase HPLC and, if necessary, was further purified by HPLC.

Enzyme Assays. The activity of RMCP I was assayed by using benzoyltyrosine ethyl ester (Bz-Tyr-OEt) as a substrate (5). Mast cell carboxypeptidase A (CPA)-like activity was assayed with benzoylglycylphenylalanine (Bz-Gly-Phe) (5).

Inactivation of Proteases by Specific Inhibitors. Free or granule-bound RMCP I was inhibited by incubation for 1 hr at room temperature in 1 mM diisopropyl phosphorofluoridate in 50 mM Tris·HCl, pH 8.0. Excess reagent was removed from the purified protease by dialysis and from the granules by repeated resuspension in 3 mM potassium phosphate buffer, pH 8.0, followed by centrifugation. CPA-like activity associated with the mast cell granules was completely inhibited after incubation for 1 hr in 3 mM potassium phosphate buffer, pH 8.0, containing 10 mM *o*-phenanthroline. Granules

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MMC, mucosal mast cells; CTMC, connective tissue mast cells; RMCP I, rat mast cell protease I; RMCP II, rat mast cell protease II; CPA, carboxypeptidase A.

could be stored up to several months at 4°C in the presence of the inhibitor without any loss of RMCP I activity.

Reduction and Carboxymethylation of Substrates. Substrates containing disulfide bonds were incubated in the presence of a reducing agent and *S*-alkylated by iodoacetic acid (11).

Proteolytic Degradation of Substrates. Granule-associated RMCP I, free RMCP I, and bovine α-chymotrypsin (the latter two in solution) were compared to each other in their ability to degrade protein and peptide substrates of known amino acid sequences. Generally, digestions were in 3 mM potassium phosphate buffer, pH 8.0, at 37°C for various periods of time (1 min to 24 hr). In some cases, digestions were carried out in 10 mM sodium phosphate buffer containing 0.15 M NaCl (PBS), pH 7.2. Enzyme-to-substrate molar ratios varied from 1:60 to 1:1030, depending on the experiment. Whenever the activities of two or more enzymes toward the same substrate were compared, the reaction mixtures contained the same concentration of enzyme activity as measured by esterase assay. At appropriate times, the reactions were stopped by adding 12 M HCl to lower the pH to below 3. To test the integrity of the granules, they were removed after various times of digestion by centrifugation and the supernatant fluids were assayed for soluble RMCP I by using Bz-Tyr-OEt or the oxidized B chain of insulin as a substrate.

Isolation of Degradation Products. Samples of digested substrates were centrifuged at 5000 × *g* for 5 min to remove granules. The supernatant fluids were adjusted to 0.1% trifluoroacetic acid with anhydrous trifluoroacetic acid and the supernatants were subjected to HPLC on a reverse-phase SynChropak RP-P column (4.1 × 250 mm) (SynChrom, Linden, IN). A linear gradient was generated, at a flow rate of 2 ml/min, consisting of buffer A (0.1% trifluoroacetic acid) and buffer B (acetonitrile containing 0.08% trifluoroacetic acid). The column effluent was monitored at 206 nm, fractions were collected, and those containing peptides were lyophilized.

Amino Acid Analysis. Purified peptide fractions were hydrolyzed at 110°C for 24 hr in evacuated and sealed tubes containing 6 M HCl. Amino acid analyses were performed by the Pico·Tag method (Waters Associates) (12).

RESULTS

An analysis of the specificities of RMCP I and α-chymotrypsin toward 12 polypeptide substrates of known amino acid sequence is given in Table 1, which lists the major differences in specificities. A total of 42 different peptide bonds were hydrolyzed after prolonged digestion of substrates (up to 20 hr) by either RMCP I or α-chymotrypsin. Under the stated conditions, 28 types of bonds were completely hydrolyzed by both proteases and one bond was partially hydrolyzed. Additionally, 13 different bonds were either partially hydrolyzed or not cleaved at all by one or the other enzyme (Table 1). For example, the Leu-Gly bond between residues 10 and 11 in histone H4 was cleaved by RMCP I but not by chymotrypsin, and the Met-Ala bond between residues 17 and 18 in vasoactive intestinal peptide and the Phe-Gly bond between residues 8 and 9 in substance P were hydrolyzed by chymotrypsin but not by RMCP I. In general, peptide bonds consisting of Leu-Xaa were hydrolyzed more readily by RMCP I than by chymotrypsin.

The degradation products obtained by incubation of angiotensin I for 20 hr with RMCP I or chymotrypsin were identical. The amino acid compositions of the fragments indicated that the substrate was cleaved by both enzymes at the bonds shown below at the arrows:

↓ ↓
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu.

Table 1. Differences in specificities of RMCP I and α-chymotrypsin toward polypeptide substrates

Cleavage site* P_2-P_1-P_1'	Substrate	Extent of hydrolysis† RMCP I	Chymotrypsin
Phe-Typ-Lys	CM-somatostatin	P	C
Gly-Phe-Phe	Insulin B chain	C	P
Gly-Phe-Ser	Bradykinin	P	C
Phe-Phe-Tyr	Insulin B chain	N	C
Phe-Phe-Gly	Substance P	N	P
Cys-Tyr-His	CM-erabutoxin B	C	P
Pro-Tyr-Ile	Neurotensin	C	T
Ala-Leu-Ile	Melittin	C	P
Ala-Leu-Tyr	Insulin B chain	C	P
Val-Leu-Lys	Melittin	C	P
Gly-Leu-Gly	Histone H4	C	N
Lys-Leu-Ser	CM-erabutoxin	C	P

Results were based on the amino acid compositions and HPLC patterns of purified degradation products obtained from prolonged digestion (up to 20 hr) of substrates. CM, carboxymethyl.
*Cleavage site of substrates as designated by Schechter and Berger (13).
†Extent of peptide bond hydrolysis: C, complete; P, partial; T, trace; N, none.

To determine if angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) was formed initially as an intermediate product, digestion was limited to 30 min and the molar ratio of enzyme to substrate was reduced approximately 10-fold. Although the fragments derived from each digest eluted from the HPLC column at identical times (Fig. 1), peaks D_c and D_R, which were not observed in the prolonged incubations, contained different peptides. In the case of chymotrypsin, the amino acid composition of fraction D_c corresponded to angiotensin II. This observation was consistent with the demonstration that purified angiotensin II eluted at an identical position as fraction D_c (Fig. 1). In contrast, fraction D_R obtained from the digestion of angiotensin I with RMCP I contained the six-residue peptide Ile-His-Pro-Phe-His-Leu and no detectable angiotensin II. Fractions A_c and A_R contained the dipeptide His-Leu, fraction B_c and B_R contained the tetrapeptide Ile-His-Pro-Phe.

To compare the substrate specificity of purified, soluble RMCP I with that of granule-bound enzyme, without interference by carboxypeptidase, isolated mast cells were incubated in 3 mM potassium phosphate, pH 8.0, containing 10 mM *o*-phenanthroline. This treatment completely inhibited the CPA without any loss in Bz-Tyr-OEt hydrolyzing activity.

Seventeen different polypeptides of various sizes and charges were incubated at 37°C for 30 min with purified or granule-bound RMCP I at enzyme-to-substrate molar ratios of 1:880 to 1:9800. With the notable exception of bradykinin, which was completely resistant, substrates of the size of hen egg white lysozyme (14,388 Da) and smaller were completely hydrolyzed by both free and granule-bound RMCP I (Table 2). No detectable activity, toward either Bz-Tyr-OEt or the B chain of oxidized insulin, was released into solution from granules during any of the experiments. Four larger polypeptides, apomyoglobin (17,199 Da), β-lactoglobulin (18,363 Da), carbonic anhydride (30,000 Da), and ovalbumin (43,500 Da), were not hydrolyzed by the granules, even after incubation for 2 hr, whereas purified RMCP I hydrolyzed these substrates completely within 30 min.

Incubation of granules with 1 mM diisopropyl phosphorofluoridate at room temperature for 1 hr abolished the capacity of granule-bound RMCP I to hydrolyze Bz-Tyr-OEt or to degrade the B chain of oxidized insulin. In contrast, incubation of granules with normal rat serum or purified α_1-anti-

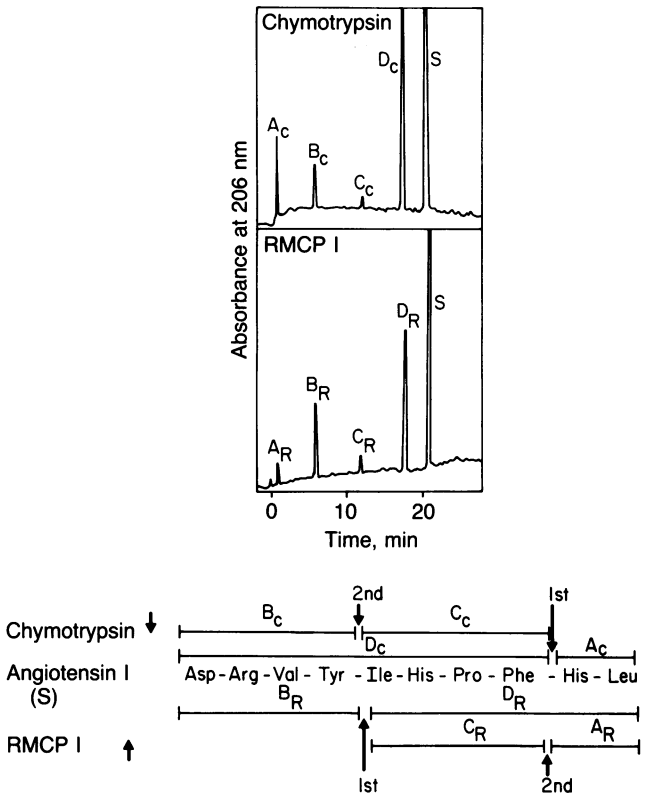


FIG. 1. Comparison of the digestion products obtained after 30-min incubation of angiotensin I with either α-chymotrypsin (*Upper*) or RMCP I (*Lower*). Incubation and HPLC separation conditions are described under *Materials and Methods*. Purified degradation peptides were characterized by amino acid composition analysis and the identities of the peptides are indicated by the schematic below the HPLC profiles.

trypsin, both of which inhibit the activity of purified RMCP I, had no effect on the proteolytic activity of the granules.

The specificity of granule-bound RMCP I toward several of the polypeptide substrates differed in kind from that of purified RMCP I. For example, the amino acid compositions of the degradation products of the B chain of oxidized insulin generated by purified or granule-bound RMCP I (Fig. 2) indicated the following degradation patterns:

Table 2. Summary of activity of granule-bound RMCP I toward polypeptide substrates

Substrate	Molecular mass, Da	Hydrolyzed?
Bradykinin*	1,024	No
Angiotensin I	1,278	Yes
Substance P	1,348	Yes
Somatostatin	1,628	Yes
Neurotensin	1,673	Yes
Vasoactive intestinal peptide	3,326	Yes
Insulin B chain†	3,496	Yes
Human pancreatic peptide	4,182	Yes
Gastric inhibitory peptide	4,974	Yes
HVWF peptide‡	5,200	Yes
Histone H4	11,300	Yes
Ribonuclease	13,690	Yes
Lysozyme (hen)	14,388	Yes
Apomyoglobin	17,199	No
β-Lactoglobulin	18,363	No
Carbonic anhydrase	30,000	No
Ovalbumin	43,500	No

Incubation and assay conditions are described in *Methods and Materials*. All substrates were readily hydrolyzed by pure RMCP I under similar conditions.
*Incubated for 4 hr.
†Prepared from oxidized insulin.
‡A purified peptide prepared in our laboratory by cyanogen bromide digestion of human von Willebrand factor.

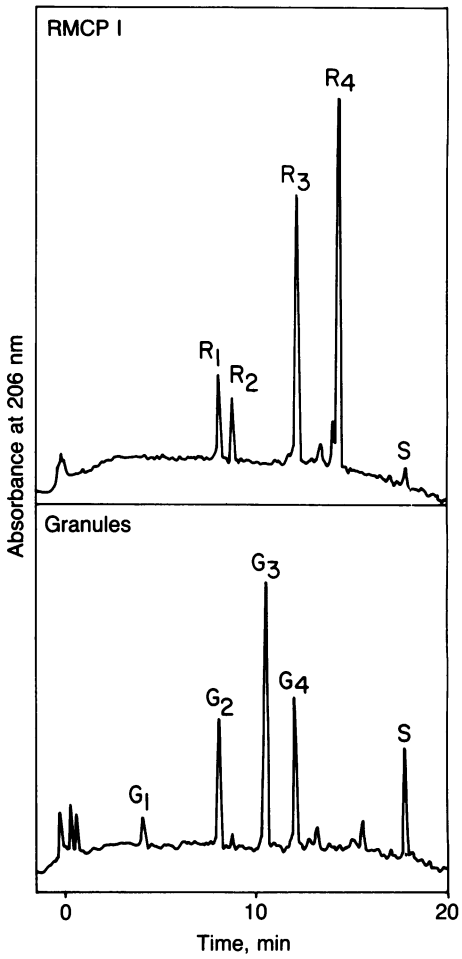
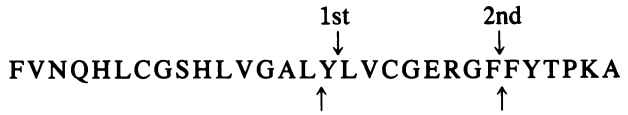


FIG. 2. Comparison of the digestion products obtained after 2-hr incubation of the B chain of oxidized insulin with either purified RMCP I (*Upper*) or granule-bound RMCP I (*Lower*). Isolated granules were treated with 10 mM *o*-phenanthroline to inhibit the CPA activity. Purified degradation peptides were identified on the basis of their amino acid compositions. S, uncleaved B chain.



Downward-pointing arrows represent cleavage by soluble RMCP I; upward-pointing arrows, granule-bound RMCP I. The cleavage of the two bonds by granule-bound RMCP I appeared to occur at about the same rate, whereas cleavage of the Y-L (Tyr-Leu) bond by purified RMCP I was more rapid than that of the F-F (Phe-Phe) bond.

Similar differences between purified and granule-bound RMCP I specificity toward other substrates tested were observed by HPLC analyses, but the nature of these differences was not determined.

DISCUSSION

The region of peptide substrates that interacts with the binding/catalytic site of serine proteases has been designated as P_2-P_1-P_1'. The substrate binding is primarily determined by

the P_1 residue, and the bond hydrolyzed is formed by the residues P_1 and P_1' (13). Many of the peptide bonds hydrolyzed by either RMCP I or α-chymotrypsin contained the same residue at the P_1 position. However, the results summarized in Table 1 indicate that, compared to the activity of chymotrypsin, RMCP I is more likely to hydrolyze peptide bonds that have hydrophobic or nonpolar residues at positions P_2 and P_1' in addition to a hydrophobic residue at P_1. The results are similar to those of earlier studies that examined the specificities of RMCP I, RMCP II, chymotrypsin, and cathepsin G toward *p*-nitroanilide-peptide substrates (14, 15). Additionally, the results indicate that RMCP I shows a greater preference than does chymotrypsin for leucine residues at the P_1 position. Interestingly, the specificity of RMCP I appears to restrict peptide bond cleavage between two hydrophobic residues such as Phe-Phe-Xaa or Phe-Trp-Xaa, in contrast to the activity of chymotrypsin, which also would cleave the Phe-Xaa and Trp-Xaa bonds. This strongly suggests a significant interaction between RMCP I and a hydrophobic residue at P_1' of the substrate, which may not be so for chymotrypsin.

In the present studies the mast cell secretory granules lacked membranes, histamine, serotonin, and other loosely associated substances. Most of the protein, including all of the RMCP I and CPA, remained associated with proteoglycan as insoluble and stable granules when resuspended in either 3 mM potassium phosphate buffer, pH 8.0, or PBS, pH 7.2, at 4°C for up to several months. None of the RMCP I dissociated from the granules, and the granule-associated RMCP I activity was completely stable during this period of time. The stability of RMCP I within the granule is in striking contrast to the rapid loss of activity, in just a few days, due to autolysis of purified RMCP I in solution, even at 4°C (5). Thus, association of RMCP I with granule-heparin appears to prevent destructive interaction among protease molecules. Similarly, the mast cell CPA is completely stable when bound within the granule.

The results of this study demonstrate clearly that granule-bound RMCP I is nearly as efficient as purified, soluble RMCP I in hydrolyzing relatively large polypeptides. Any detectable proteolytic capacity of the granules could be attributed to RMCP I or CPA, consistent with previous observations that these are the principal proteases contained in mast cell granules.

In the case of small peptides such as angiotensin I, the specificity of granule-bound RMCP I was identical to that of purified RMCP I. However, when larger peptides, such as the B chain of oxidized insulin, were used as substrates, different fragments were generated, depending on whether the protease was soluble or bound to granules (Fig. 2). The reason for the change in specificity of RMCP I must await the results of further investigation.

Surprisingly, the hypotensive peptide bradykinin was not hydrolyzed by granule-bound RMCP I even though the peptide contains two peptide bonds that were slowly hydrolyzed during prolonged incubation with purified soluble RMCP I. This result, together with the observation that granule-bound RMCP I hydrolyzed angiotensin I without generating the potent vasoconstriction peptide angiotensin II, suggests a functional role of granule-bound RMCP I—i.e., to regulate, after exocytosis, the levels of the vasoactive peptides angiotensin I/II and bradykinin so as to result in an excess of bradykinin and a general hypotensive state. Interestingly, in contrast to the action of RMCP I, human mast cell chymase apparently does generate angiotensin II from angiotensin I (16).

The results of this study indicate that polypeptides as large as hen egg white lysozyme (14,388 Da) are readily hydrolyzed by granule-bound RMCP I, whereas larger molecules such as apomyoglobin (17,199 Da) and β-lactoglobulin (18,363 Da) are resistant to proteolytic attack. The net charge of the polypeptides does not affect this selectivity since, at the pH of incubation, positively charged vasoactive intestinal peptide, neutrally charged glucagon, and the negatively charged B chain of oxidized insulin all were rapidly hydrolyzed by granule-bound RMCP I. That the size of the polypeptide limits access to granule-bound RMCP I is also supported by the observation that neither normal rat serum nor α_1-antitrypsin, both effective inhibitors of purified RMCP I, apparently, is packaged internally within granules in a manner that precludes any interaction with polypeptides larger than approximately 20,000 Da. RMCP I, and perhaps also CPA, would appear to be entrapped within a porous and rigid granule matrix formed by high molecular mass (>700,000 Da) heparin. If RMCP I were located fully exposed on the surface of the granule, one would anticipate at least partial degradation of the larger polypeptide substrates that have been tested.

In any event, the results of this study suggest at least two consequences of mast cell granule secretion into extracellular fluids. First, the vast majority of proteins present in plasma, in the extracellular matrix, and on cell surfaces would be too large to interact with granule-bound RMCP I. Second, expression of RMCP I activity within the secreted granules is likely to continue unabated until the granules are either recovered by mast cells or engulfed by phagocytes, since protease inhibitors of blood fail to inactivate granule-associated RMCP I. These consequences would be irrelevant if RMCP I were solubilized by extracellular fluids. The present results conclusively demonstrate that under physiological conditions of ionic strength and pH no detectable RMCP I activity is released from the granules into the fluid phase.

Overall the results of this study clearly demonstrate the feasibility and appropriateness of using intact secretory granules rather than purified enzymes for future studies in this area.

This work was supported by Grants HL-36114 and GM-15731 from the National Institutes of Health.

1. Tas, J. & Berndsen, R. G. (1977) *J. Histochem. Cytochem.* **25,** 1058–1062.
2. Ennerbäck, L. (1966) *Acta Pathol. Microbiol. Scand.* **66,** 303–312.
3. Woodbury, R. G., Gruzenski, G. M. & Lagunoff, D. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2785–2789.
4. Woodbury, R. G., Everitt, M., Sanada, Y., Katunuma, N., Lagunoff, D. & Neurath, H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5311–5313.
5. Woodbury, R. G., Everitt, M. T. & Neurath, H. (1981) *Methods Enzymol.* **80,** 588–609.
6. Woodbury, R. G. & Neurath, H. (1980) in *Metabolic Interconversion of Enzymes*, ed. Holzer, H. (Springer, Berlin), pp. 145–158.
7. Austen, K. F. (1978) *J. Immunol.* **121,** 793–805.
8. Shick, B., Austen, K. F. & Schwartz, L. B. (1984) *J. Immunol.* **132,** 2571–2577.
9. Lagunoff, D. & Pritzl, P. (1976) *Arch. Biochem. Biophys.* **173,** 554–563.
10. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
11. Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238,** 622–627.
12. Bidlingmeyer, B. A., Cohen, S. A. & Tarvin, T. L. (1984) *J. Chromatogr.* **336,** 93–104.
13. Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27,** 157–162.
14. Yoshida, N., Everitt, M. T., Woodbury, R. G., Neurath, H. & Powers, J. C. (1980) *Biochemistry* **19,** 799–804.
15. Powers, J. C., Tanaka, T., Harper, J. W., Minematsu, Y., Barker, L., Lincoln, D., Crumley, K. V., Fraki, J. E., Schechter, N. M., Lazarus, G. G., Nakajima, K., Nakashino, K., Neurath, H. & Woodbury, R. G. (1985) *Biochemistry* **24,** 2048–2058.
16. Reilly, C. F., Tewksbury, D. A., Schechter, N. M. & Travis, J. (1982) *J. Biol. Chem.* **257,** 8619–8622.