Pergamon

1357-2725(95)00158-1

Int. J. Biochem. Cell Biol. Vol. 28, No. 4, pp. 451–456, 1996

1357-2725/96 $15.00 + 0.00

Peptide Degradation: Effect of Substrate Phosphorylation on Aminopeptidasic Hydrolysis

PEDRO FERNÁNDEZ MURRAY, PABLO HAMMERSCHMIDT, ANDREA SAMELA, SUSANA PASSERON*

Cátedra de Microbiología, Facultad de Agronomía, Universidad de Buenos Aires and CONICET, Avda San Martín 4453, 1417 Buenos Aires, Argentina

The effect of substrate phosphorylation on the susceptibility to exopeptidasic attack by leucyl aminopeptidase of swine kidney, alanyl aminopeptidase from human liver and aminopeptidase N of *Escherichia coli* was investigated using a synthetic heptapeptide (L-R-R-A-S-L-G) and its phosphorylated derivative. The enzyme-catalyzed products were analyzed by thin layer chromatography and electrophoresis. The sensitivities of peptide and phosphopeptide to leucyl aminopeptidase digestion were then compared. Data obtained indicated that when phosphopeptide was used as substrate one main product accumulated, which corresponded to the fragment A-S(P)-L-G, while unphosphorylated peptide was completely degraded to its constituent amino acids. Identical results were obtained using aminopeptidase N of *E. coli*. Using alanyl aminopeptidase as enzyme, the results obtained were essentially similar, since the exopeptidasic activity on the phosphorylated peptide was strongly hampered in the vicinity of phosphoseryl residue leading to accumulation of the same phosphorylated product, although this enzyme could not completely degrade the unphosphorylated peptide. It was concluded that phosphorylation of substrates does effect enzymic degradation of proteins. Copyright © 1996 Elsevier Science Ltd.

Keywords: Aminopeptidases Phosphopeptides Peptide degradation

Int. J. Biochem. Cell Biol. (1996) **28**, 451–456

1. INTRODUCTION

Intracellular proteolysis represents an essential mechanism in the regulation of the cellular function at the post-traslational level (Bond and Butler, 1987; Wolf, 1992). In general, total hydrolysis of proteins requires the prior chain-cutting action of the endopeptidases, but the action of exopeptidases, especially aminopeptidases, is essential for complete breakdown of protein substrates to render free amino acids (Botbol and Scornik, 1989; Arbesú *et al.*, 1991).

Accumulated evidence indicates that several kinds of covalent modification of proteins greatly alter their sensitivity to endoproteolytic degradation (Stadtman, 1990). Among these post-translational modifications are phosphorylation–dephosphorylation of seryl or threonyl residues (Garland and Nimmo, 1984; Chen and Stracher, 1989). Benore-Parsons *et al.* (1989), using kemptide (L-R-R-A-S-L-G) and its phosphorylated derivative, have demonstrated that phosphorylation of the seryl residue drastically reduced the cleavage of kemptide by endopeptidases such as trypsin and kallikrein. Also, Bramucci *et al.* (1992) have reported that phosphorylation of epidermal mitosis inhibiting pentapeptide protected the peptide from hydrolysis by a serum enzyme. However, at present, little is known about the effect of substrate phosphorylation on the action of exopeptidases.

We have previously demonstrated that exhaustive phosphokemptide hydrolysis by the mayor aminopeptidase from the fungus

*To whom correspondence should be addressed. Fax: 54-1-523-4936; e-mail: passeron@fotgar.uba.an.

Abbreviations: kemptide: L-R-R-A-S-L-G; LAP: leucyl aminopeptidase (EC 3.4.11.1); AAP: alanyl aminopeptidase (EC 3.4.11.14); APN: aminopeptidase N.

Received 10 October 1995; accepted 10 October 1995

Saccobolus platensis led to accumulation of the A-S(P)-L-G fragment, while unphosphorylated kemptide was completely degraded to its constituent amino acids (Fernández Murray *et al.*, 1992; Fernández Murray and Passeron, 1994). Moreover, using a heterogeneous population of peptides and phosphopeptides as substrates we have shown (Fernández Murray and Passeron, 1994) that after exhaustive aminopeptidasic degradation no detectable amounts of phosphoamino acids were produced and an enzyme-resistant peptide population accumulated. The presence of phosphoamino acyl residue accounted for the aminopeptidase-resistant character of a fraction of these peptides. The remainder refractory peptides exhibited at its N-termini the X-P motif. These results, to our knowledge, constituted the first report on the impairment of aminopeptidase activity by substrate phosphorylation and prompted us to extend these studies to other well-characterized enzymes such as leucyl aminopeptidase (LAP) from swine kidney, alanyl aminopeptidase (AAP) from human liver and aminopeptidase N (APN) from *Escherichia coli*, which share with the fungal aminopeptidase a broad substrate specificity.

The results obtained indicate that phosphorylation of the serine residue in a model heptapeptide, kemptide, prevents its complete hydrolysis by the three aminopeptidases. This finding suggests that dephosphorylation must occur before the complete degradation of oligopeptides to amino acids.

2. MATERIALS AND METHODS

Kemptide phosphorylation

Kemptide was routinely phosphorylated with [γ-^{32}P]ATP and the catalytic subunit of cyclic AMP-dependent protein kinase as described previously (Fernández Murray *et al.*, 1989). Purification of [^{32}P]phosphokemptide was carried out as described in a preceding paper (Fernández Murray *et al.*, 1992).

Purification of leucyl aminopeptidase

Leucyl aminopeptidase, obtained from Sigma Chemical Co. (type III-CP, lots No. 38 F 8080 and 102 H 8035), was purified by affinity chromatography on a leucyl–glicyl–AH–Sepharose column and its Zn^{2+} content restored following the protocol described by Van Wart and Lin (1981). A typical preparation of this enzyme after this procedure exhibited an activation of 7–10-fold after preincubation during 4 h at 37°C with 5 mM Mg^{2+} and had a specific activity of about 0.35 units per milligram of protein. One enzyme unit was defined as the amount of enzyme that released 1 μmol of *p*-nitroaniline per minute at 22°C. The measurement of leucyl aminopeptidase using L-leucyl-*p*-nitroanilide as substrate, was carried out essentially as described by Van Wart and Lin (1981).

Purification of alanyl aminopeptidase

Normal human liver samples were obtained from residual parts of surgically excised materials remaining after microscopic examination, and were stored at − 70°C until use. Alanyl aminopeptidase was purified to near homogeneity as described by Garner and Behal (1975). A typical preparation, with a specific activity of about 65 units per milligram of protein, exhibited a 2-fold activation when assayed with 2 mM Co^{2+}. A colorimetric assay was used to measure alanyl aminopeptidase activity, using L-alanyl-β-naphtylamide as substrate (Garner and Behal, 1975). One enzyme unit was defined as the amount of enzyme that liberated 1 μmol of β-naphtylamine per minute at 37°C.

Purification of aminopeptidase N

Aminopeptidase N from *E. coli* K12 strain was purified to near homogeneity following the protocol of McCaman and Villarejo (1982). The purification was performed up to the Sephadex G-150 column chromatography step and rendered an enzyme preparation essentially free of other contaminant peptidase activities, with a specific activity of about 5 units per milligram of protein. Aminopeptidase N was measured using L-alanyl-*p*-nitroanilide as substrate, as described by McCaman and Villarejo (1982). One enzyme unit was defined as the amount of enzyme that catalyzed the release of 1 μmol of *p*-nitroaniline per minute at 37°C.

Amino acid oxidase–peroxidase assay

Amino acids released from kemptide were subjected to oxidative deamination by L-amino acid oxidase and the appearance of hydrogen peroxide was followed spectrophotometrically by measuring the oxidation of *o*-dianisidine (Logan *et al.*, 1983).

Thin layer chromatography (TLC)

Amino acids released from kemptide were identified by ascending chromatography on cellulose plates (20 × 20 cm) using pyridine:dioxane:ammonium hydroxide:water (35:35:15:15) (v/v) as solvent. The amino acids were detected by spraying the plates with ninhydrin 0.25% (w/v) in acetone, followed by heating.

Thin layer electrophoresis

Electrophoresis was run on cellulose plates (20 × 20 cm) at pH 3.5 (10:100:890; pyridine:glacial acetic acid: water) (v/v) for 2 h at 300 V. Unlabeled phosphoserine, used as internal standard, was visualized with ninhydrin. Radioactive spots were located by autoradiography.

Hydrolysis of phosphokemptide and purification of the main product

The hydrolysis of [^{32}P]phosphokemptide (100 μM; 100–200 cpm/pmol) was carried out in a final volume of 1750 μl under the experimental conditions given in Section 3 (Table 1). After incubation, the reaction was stopped by adding 750 μl of glacial acetic acid, and the mixture was applied to a column containing 2 ml of phosphocellulose resin equilibrated with 30% (v/v) acetic acid. The products of aminopeptidase-catalyzed hydrolysis of [^{32}P]-phosphokemptide eluted with 30% (v/v) acetic acid (3 ml), whereas intact [^{32}P]phosphokemptide and the first product, devoid of N-terminal leucine, remained bound to the resin. The acetic acid in the eluate was removed by evaporation and washing twice with water. After drying, the residue was dissolved in 400 μl of 20 mM ammonium acetate, pH 6.8, and applied to a column of AG 1 × 8 (1 ml of resin) equilibrated with 20 mM ammonium acetate. The column was washed successively with 3 ml each of 20, 200 and 700 mM ammonium acetate. The eluates were separately concentrated by freeze-drying and appropriate aliquots were analyzed by electrophoresis as described above. The main product eluting at 700 mM ammonium acetate was used for amino acid analysis.

Amino acid analysis

Amino acid analysis was carried out with a 119 CL Beckman amino acid analyzer. When indicated, the samples were previously hydrolyzed with 6 N HCl for 24 h at 110°C.

Chemicals

Freeze-dried kemptide was obtained from Peninsula Laboratories, Inc., and stored at − 20°C. [γ-^{32}P]ATP was obtained from New England Nuclear. AG 1 × 8 resin was from Bio-Rad. TLC cellulose plates, chromogenic amino acids, the catalytic subunit of cyclic AMP-dependent protein kinase (bovine heart), L-amino acid oxidase from *Crotalus adamanteus* (type I), peroxidase (horseradish) and leucyl aminopeptidase (type III-CP) were purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

3. RESULTS

Kemptide hydrolysis

Time course of kemptide hydrolysis by LAP was followed by TLC analysis and by the production of free amino acids with the amino acid oxidase–peroxidase assay. It was estimated

Table 1. Amino acid analysis of phosphokemptide and its enzymatic products[a]

	Amino acid content					
Aminopeptidase treatment[b]	L	R	A	S	G	Proposed sequence
—	1.9	2.0	1.0	0.8	1.2	L-R-R-A-S(P)-L-G
Leucyl aminopeptidase	1.0	—	1.0	0.7	0.9	A-S(P)-L-G
Alanyl aminopeptidase	1.1	—	1.0	0.8	1.0	A-S(P)-L-G
Aminopeptidase N	1.0	—	1.0	0.8	1.1	A-S(P)-L-G

[a] Results are molar ratio of amino acids expressed relative to alanine.

[b] [^{32}P]Phosphokemptide (175 nmol; 100–200 cpm/pmol) was incubated in a reaction mixture (1.75 ml) containing (i) 0.3 milliunits of leucyl aminopeptidase (previously preincubated at 37°C for 4 h with 5 mM $MgCl_2$); 10 mM Tris-HCl buffer, pH 8.8; 5 mM $MgCl_2$ for 60 min at 22°C or (ii) 110 milliunits of alanyl aminopeptidase; 25 mM potassium phosphate buffer, pH 6.8; 2 mM $CoCl_2$ for 60 min at 37°C or (iii) 0.5 units of aminopeptidase N; 25 mM sodium phosphate buffer, pH 7.5, for 60 min at 37°C. At the end of the incubation period, the mixtures were processed as described under Materials and Methods.

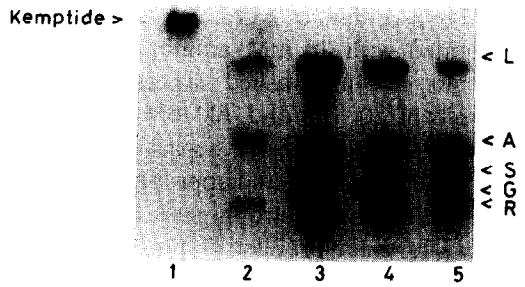

Fig. 1. Kemptide hydrolytic products by several aminopeptidase treatments. Kemptide (4 mM) was incubated for 90 min with: (i) 70 milliunits of alanyl aminopeptidase in a final volume of 50 µl, containing 25 mM potassium phosphate buffer, pH 6.8, and 2 mM $CoCl_2$ at 37°C; (ii) 350 milliunits of aminopeptidase N in 50 µl containing 25 mM sodium phosphate buffer, pH 7.5, at 37°C and (iii) 0.6 milliunits of leucyl aminopeptidase (previously preincubated for 4 h at 37°C with 5 mM $MgCl_2$) in a final volume of 50 µl 5 mM Tris-HCl buffer, pH 8.8; 5 mM $MgCl_2$ at 22°C. At the end of incubation period, 30 µl aliquots were withdrawn and heated at 95°C for 3 min. After centrifugation at 10,000 rpm for 10 min, appropriate aliquots of the supernatants were loaded and chromatographed as described under Materials and Methods. Lane 1: kemptide standard; lane 2: alanyl aminopeptidase activity products (2 µl of supernatant); lane 3: aminopeptidase N activity products (5 µl of supernatant); lane 4: leucyl aminopeptidase activity products (4 µl of supernatant); lane 5: reference amino acids. The spots were revealed with ninhydrin.

that the rate of kemptide hydrolysis was approximately 100-fold faster than that measured on the *p*-nitroanilide derivative of leucine. In addition, a uniform increase of all constituent amino acids during the incubation was observed, rather than a sequential release of free amino acids, as might be expected (data not shown). Since LAP exhibits a low rate of hydrolysis of peptides that possess basic residues at position P_1 and/or P'_1* (Burley *et al.*, 1991), these results could be explained considering that the peptides produced after the removal of the N-terminal leucine and arginine are much better substrates than the intact kemptide or than fragments containing one or two arginyl residues at the N-termini.

Figure 1 shows the amino acid patterns produced by exhaustive digestion of kemptide by AAP, APN, and LAP. As can be seen, APN and LAP hydrolyzed the substrate, rendering the constituent amino acids (lanes 3 and 4, respectively), while AAP could only release leucine, arginine and alanine (lane 2). This result was confirmed by amino acid analysis of the products: only leucine, arginine and alanine were quantitatively detected after exhaustive kemptide incubation with AAP, suggesting that this enzyme possesses a very low hydrolytic rate on S–L peptidic bond. In agreement, Little *et al.* (1976) reported that among several dipeptide substrates, S–G exhibited the lowest rate of hydrolysis by AAP.

Phosphokemptide hydrolysis

Figure 2 shows the time course of [^{32}P]phosphokemptide hydrolysis by LAP. As can be seen (lane 2), after 10 min of incubation phosphokemptide and two additional phosphorylated products, with less cationic character, were detected. At 15 min of incubation, phosphokemptide and the slow migrating product almost disappeared (lane 3), and at 30 min of incubation the fast migrating product accumulated (lane 4). This phosphorylated fragment, which exhibits a different mobility of phosphoserine, was the unique product detected even after long incubation periods (not shown). These results indicate that LAP could hydrolyze the phosphorylated substrate leading to accumulation of one main product.

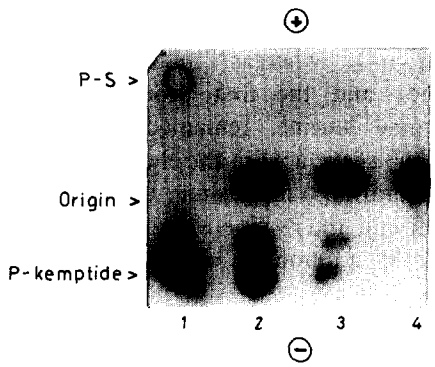

Fig. 2. Time course analysis of phosphokemptide degradation by leucyl aminopeptidase. [^{32}P]Phosphokemptide (100 µM; 100–200 cpm/pmol) was incubated at 22°C with 0.02 milliunits (previously preincubated at 37°C for 4 h with 5 mM $MgCl_2$) in a final volume of 100 µl containing 5 mM Tris-HCl buffer, pH 8.8; 5 mM $MgCl_2$. At the times indicated 20 µl aliquots were withdrawn and boiled. After centrifugation at 10,000 rpm for 10 min, 10 µl of supernatant were analyzed by electrophoresis as described under Materials and Methods. Lane 1: [^{32}P]phosphokemptide and phosphoserine standards; lanes 2–4: 10, 15 and 30 min of incubation. Radioactive compounds were detected by autoradiography; phosphoserine was revealed with ninhydrin. P-kemptide: [^{32}P]phosphokemptide; P-S: phosphoserine.

*Following the convention of Schechter and Berger (1967), the amino acid side chains P1 and P'1 binding to sites S1 and S'1 on the enzyme, respectively, where the cleavage site is between S1 and S'1.

In order to identify this phosphorylated product, a large amount was generated by exhaustive degradation of [^{32}P]phosphokemptide by LAP under the experimental conditions given in footnote (b) of Table 1. The phosphorylated product was purified by phosphocellulose and AG 1 × 8 column chromatography, as described in Section 2. Amino acid analysis of the purified fragment (see Table 1) allowed its identification as A-S(P)-L-G.

Exhaustive degradation of [^{32}P]phosphokemptide by AAP and APN rendered phosphorylated products with electrophoretic mobilities similar to the LAP accumulated peptide (data not shown). These two fragments were purified as described in Section 2 and their amino acid analyses (Table 1) revealed that their sequences correspond to the same peptide produced by LAP activity.

4. DISCUSSION

Post-translational modifications of intracellular proteins play a vital role in cellular homeostasis. These modifications are involved in a variety of functions such as protein traffic to various cellular compartments as well as protein folding and turnover. Accumulated evidence indicates that phosphorylation–dephosphorylation of proteins deeply influences their sensitivity to endoproteolytic degradation (Garland and Nimmo, 1984; Chen and Stracher, 1989; Bramucci *et al.*, 1992; Rittenhouse *et al.*, 1987).

It would be expected, therefore, that aminopeptidase activity could also be affected by the presence of a phosphoamino acyl residue in the peptidic substrate. Previous work from our laboratory demonstrated that kemptide was readily degraded to its constituent amino acids by the major aminopeptidase of *S. platensis* (Fernández Murray *et al.*, 1992), while phosphorylation of the seryl residue severely hampered enzyme action, leading to accumulation of the A-S(P)-L-G fragment (Fernández Murray and Passeron, 1994).

In this paper we present evidence indicating that the impairment of aminopeptidase activity by substrate phosphorylation can be extended to three other well-known enzymes: leucyl aminopeptidase (LAP) from swine kidney, alanyl aminopeptidase (AAP) from human liver and aminopeptidase N (APN) from *E. coli*. The fact that these three enzymes could hydrolyze phosphokemptide, leading to accumulation of the same phosphorylated fragment, v.g. A-S(P)-L-G, indicates that the blockade of aminopeptidasic hydrolysis occurs on the N-terminal removable residue adjacent to the phosphoseryl residue. Although LAP and APN are members of different metallo-aminopeptidase families (Rawlings and Barrett, 1993), our results suggest that both enzymes might possess a common structural/functional feature.

Among the many suggested functions of aminopeptidases (Taylor, 1993a,b), the final degradation of proteins to amino acids is prominent. Thus, Botbol and Scornik (1989), using bestatin, an aminopeptidase inhibitor, demonstrated the participation of these enzymes in intracellular protein degradation. Also, the fact that cytosolic carboxypeptidases have not been found in yeast (Suárez Rendueles and Wolf, 1988; Jones, 1991) or mammalian cells (Mc Donald and Barrett, 1986) suggests an essential role for aminopeptidases in the final step of cytoplasmic degradation of proteins. Taking into account the experimental data provided here, we are tempted to speculate that in-vivo degradation of peptides should require prior removal of phosphate, among other post-translational modifications.

Work is in progress aimed at (i) investigating the effect of substrate phosphorylation on the kinetic constants of aminopeptidase hydrolysis and (ii) to determine if the resistant character of the phosphorylated peptide A-S(P)-L-G is due to a very low enzyme affinity constant or to a non-productive enzyme–substrate complex formation.

Acknowledgements—We are grateful to Dr Ricardo N. Farías for pithy discussion of our results and Dr E. Passeron for critically reading the manuscript. We also wish to thank the Laboratorio Nacional de Investigación y Servicios en Péptidos y Proteínas (LANAIS-PRO, CONICET-UBA) for performing amino acid analyses. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina) and from the Universidad de Buenos Aires.

REFERENCES

Arbesú M. J., Gascón S. and Suárez-Rendueles P. (1991) Isolation and characterization of *Schizosaccharomyces pombe* mutants lacking aminopeptidase activity. *Yeast* **7**, 525–531.

Benore-Parsons M., Seidah N. G. and Wennogle L. P. (1989) Substrate phosphorylation can inhibit proteolysis by trypsin-like enzymes. *Arch. Biochem. Biophys.* **272**, 274–280.

Bond S. J. and Butler P. L. (1987) Intracellular proteases. *Ann. Rev. Biochem.* **56**, 333–364.

Botbol V. and Scornik O. A. (1989) Role of bestatin-sensitive exopeptidases in the intracellular degradation of hepatic proteins. *J. Biol. Chem.* **264,** 13504–13509.

Bramucci M., Miano A. and Amici D. (1992) Epidermal inhibitory pentapeptide phosphorylated *in vitro* by calf thymus protein kinase NII is protected from serum enzyme hydrolysis. *Biochem. Biophys. Res. Commun.* **183,** 474–480.

Burley S. K., David P. R. and Lipscomb W. N. (1991) Leucine aminopeptidase: bestatin inhibition and a model for enzyme-catalyzed peptide hydrolysis. *Proc. Natl Acad. Sci. USA* **88,** 6916–6920.

Chen M. and Stracher A. (1989) *In situ* phosphorylation of platelet actin-binding protein by cAMP-dependent protein kinase stabilizes it against proteolysis by calpain. *J. Biol. Chem.* **264,** 14282–14289.

Fernández Murray P., Silberstein S., Cantore M. L. and Passeron S. (1989) A radioactive method for the measurement of trypsin and trypsin-like activities. *Anal. Biochem.* **179,** 56–59.

Fernández Murray P., Samela A. and Passeron S. (1992) Purification and characterization of an aminopeptidase from the fungus *Saccobolus platensis*. *Exp. Mycol.* **16,** 279–290.

Fernández Murray P. and Passeron S. (1994) Phosphorylated peptides can limit Saccobolus platensis aminopeptidase action. *Exp. Mycol.* **18,** 320–329.

Garland D. and Nimmo H. G. (1984) A comparison of the phosphorylated and unphosphorylated forms of isocitrate dehydrogenase from *Escherichia coli* ML308. *FEBS Lett.* **165,** 259–264.

Garner C. W. and Behal F. J. (1975) Human liver alanine aminopeptidase inhibition by amino acids. *Biochemistry* **14,** 3208–3212.

Jones E. W. (1991) Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266,** 7963–7966.

Little G. H., Starnes W. L. and Behal F. J. (1976) Human liver aminopeptidase. *Methods Enzymol.* **45,** 495–503.

Logan D. A., Naider F. and Becker J. M. (1983) Peptidases of yeast and filamentous forms of *Candida albicans*. *Exp. Mycol.* **7,** 116–126.

McCaman M. T. and Villarejo M. R. (1982) Structural and catalytic properties of peptidase N from *Escherichia coli* K-12. *Arch. Biochem. Biophys.* **213,** 384–394.

McDonald J. K. and Barrett A. J. (1986) in *Mammalian Proteases. Vol. 2, Exopeptidases*. Academic Press, Orlando, Florida.

Rawlings N. D. and Barrett A. J. (1993) Evolutionary families of peptidases. *Biochem. J.* **290,** 205–218.

Rittenhouse J., Moberly L. and Marcus F. (1987) Phosphorylation *in vivo* of yeast (*Saccharomyces cerevisiae*) fructose-1,6-bisphosphatase at the cyclic AMP-dependent site. *J. Biol. Chem.* **262,** 10114–10119.

Schechter I. and Berger A. (1967) Characterization of a pyroglutamate aminopeptidase from rat serum that degrades thyrotropin-releasing hormone. *Biochem. Biophys. Res. Commun.* **27,** 157–162.

Stadtman E. R. (1990) Covalent modifications reactions are marking steps in protein turnover. *Biochemistry* **29,** 6323–6331.

Suárez Rendueles P. and Wolf D. H. (1988) Proteinases function in yeast: biochemical and genetics approaches to a central mechanism of post-translational control in eukaryotic cell. *FEMS Microbiol. Rev.* **54,** 17–46.

Taylor A. (1993) Aminopeptidases: structure and function. *FASEB J.* **7,** 290–298.

Taylor A. (1993) Aminopeptidases: towards a mechanism of action. *Trend Biochem. Sci.* **18,** 167–172.

Van Wart H. E. and Lin S. H. (1981) Metal binding stoichiometry and mechanism of metal ion modulation of the activity of porcine kidney leucine aminopeptidase. *Biochemistry* **20,** 5682–5689.

Wolf D. H. (1992) Proteases as biological regulators. *Experientia* **48,** 117–118.