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Hydrolysis of Phosphopeptides

II. Leucine Aminopeptidase Hydrolysis of Free and O-Phosphorylated Serine Peptides

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The rates of leucine aminopeptidase hydrolysis of O-phosphorylated, O-monophenylphosphorylated, and O-pyrophosphorylated serine peptides were determined and compared with those of the corresponding phosphate-free peptides. A phosphate group near the free α-amino end, as in N-terminal O-phosphorylserine peptides, lowers the rate of hydrolysis more than 100 times, and a pyrophosphate group in the same position more than 1000 times. O-Monophenylphosphorylated peptides are hydrolyzed at about the same rate as their phosphate-free analogues.

The limited *in vitro* hydrolysis of phosphoproteins by the proteolytic enzymes occurring in the intestinal tract (pepsin, trypsin, chymotrypsin *etc.*) has been known for a long time. The phosphate-containing peptides obtained by this limited hydrolysis seem to resist further attack.[1,2] The phosphate groups in these peptides are now regarded to be esterbound to hydroxyl groups of serine and, to some extent, threonine.[2-4] It seems logical to attribute the resistance of the peptide bonds to the presence of these O-phosphoryl groups near the bonds. We have found that this is true in the case of trypsin action on some synthetic substrates: the bond between lysine and serine in a peptide derivative was easily hydrolyzed by trypsin but turned out to be resistant when the serine hydroxyl group was phosphorylated.[5] It was shown by acid-base titration, that an O-phosphorylserine residue close to lysine has a strong electrostatic effect on the ε-amino group of the latter and thereby may block this group.[6] Free ε-amino group is, however, a wellknown necessary requirement for trypsin hydrolysis. Some natural phosphopeptides have been isolated which were resistant to trypsin action although containing lysine [2,4] *within* the peptide chain. One may assume, that these peptides also have their O-phosphorylserine residues near lysine.

The natural phosphopeptides isolated do not usually contain the »aromatic» amino acids, phenylalanine, tyrosine, and tryptophane. Their resistance to

such enzymes as pepsin, chymotrypsin, and carboxypeptidase is therefore expected. One natural peptide having the C-terminal sequence . . . Tyr-Lys was, however, isolated by us from human casein after degradation with trypsin.[7] Chymotrypsin liberated lysine from this peptide, but we do not yet know how far the nearest O-phosphoryl-serine residue (of the total five such residues) is situated from the tyrosine residue.

Leucine aminopeptidase is the important exopeptidase of the small intestine.[8] Posternak and collaborators showed, that the casein-peptide O-phosphoryl-L-seryl-L-glutamic acid [9] and the four synthetic peptides [10] glycyl-(O-phosphoryl)-L-tyrosine, O-phosphoryl-L-tyrosylglycine, O-phosphoryl-L-tyrosylglycylglycine, and glycyl-(O-phosphoryl)-L-tyrosylglycine were resistant to a crude peptidase preparation from swine small intestine. The peptides were, however, hydrolyzed easily after removal of the O-phosphoryl groups by the action of a phosphatase. Posternak also showed that 6-phosphorylated 1,4-glycosides were similarly resistant to β-amylase action.[9]

Purified leucine aminopeptidase has been used for hydrolysis of the phosphopeptide Lys-Glu(NH_2)-Ile-SerP-Val-Arg, isolated from phosphorylase a.[11] Lysine and glutamine were liberated, but the bond between isoleucine and O-phosphorylserine was attacked very slowly by the enzyme.

The present paper is a part of our studies on phosphoproteins and phosphopeptides and will describe the rates of hydrolysis of synthetic peptides with purified leucine aminopeptidase.

EXPERIMENTAL

Substrates. The synthesis and analytical data for the following peptides has been published:[12,13] DL-serylglycine, glycyl-DL-serine, and their O-phosphorylated and O-monophenylphosphorylated analogues (in Ref. 12), L-seryl-L-leucine, O-phosphoryl-L-seryl-L-leucine, L-leucyl-L-serine; L-leucyl-(O-phosphoryl)-L-serine, L-seryl-L-glutamic acid, O-phosphoryl-L-seryl-L-glutamic acid, α-L-glutamyl-L-serine, and α-L-glutamyl-(O-phosphoryl)-L-serine (in Ref. 13).

The synthesis and data for the following peptides will be described separately:[14] L-isoleucyl-L-serine, L-isoleucyl-(O-phosphoryl)-L-serine, L-lysyl- L-serine, L-lysyl-(O-phosphoryl)-L-serine, α-L-aspartyl-L-serine, α-L-aspartyl-(O-phosphoryl)-L-serine, γ-L-glutamyl-L-serine, γ-L-glutamyl-(O-phosphoryl)-L-serine, O-phosphoryl-L-seryl-L-alanine, α-L-glutamyl-L-seryl-L-alanine, L-leucylglycyl-L-serine, L-leucyl-glycyl-(O-phosphoryl)-L-serine (in Ref. 14) O-pyrophosphoryl-L-seryl-L-leucine and L-leucyl-(O-pyrophosphoryl)-L-serine (in Ref. 15).

The following commercial substrates have been used: L-leucylglycine (Hofmann-La Roche, Switzerland), glycyl-L-leucine (YEDA, Israel), L-serylglycine (Mann, U.S.A.), L-seryl-L-alanine (YEDA, Israel) and glycylglycine (Hofmann-La Roche, Switzerland).

All substrates were analyzed paper-chromatographically. Their optical purity was in most cases proved by letting leucine-aminopeptidase act to 100 % hydrolysis (peptides containing DL-serine thereby gave about 50 % hydrolysis).

Enzyme. Leucine aminopeptidase was prepared according to Hill *et al.*[16] The activity $C_1 = 6$ was obtained in a volume of 36 ml and 4.5 mg protein-N/ml after the acetone-precipitation (step 6).

Hill *et al.* used zone-electrophoresis after this step. We tried instead gel filtration on a Sephadex G 75 (75 × 4 cm) column, eluting with a buffer (0.005 M Tris + 0.005 M $MgCl_2$) of pH 8.0. The active fraction (100 ml) was collected in a dialysis bag and concentrated to 11.5 ml in an air stream. The activity was then $C_1 = 5$ and the protein-N 7.55 mg/ml.

As this step did not increase the activity, we then used the method described by Folk *et al.* [17]: A column (75 × 3 cm) of DEAE-cellulose was equilibrated with 0.005 M Tris-HCl buffer of pH 8 and containing 0.005 M $MgCl_2$, the sample applied and gradient eluted to the same buffer containing 0.75 M NaCl (mixing chamber 1 l). The active peak was collected and dialysed against 0.005 M Tris-HCl containing 0.005 M $MgCl_2$. The final volume was 10 ml; protein-N = 0.88 mg/ml and the activity $C_1 = 22$. This solution was kept in small test-tubes at −20°.

Determination of hydrolysis rates. The amount of enzyme solution, calculated to give 20 − 30 % hydrolysis of a substrate in 20 min was added to a mixture of 0.020 ml of 0.025 M $MnCl_2$ and 0.050 ml of 0.5 M tris buffer pH 8.5. The mixture was diluted to 0.250 ml and left 30 min at 40° for activation.

To this enzyme solution was added a mixture of 0.2 ml of 0.025 M $MnCl_2$, 0.5 ml 0.5 M tris buffer pH 8.5, 1.0 ml 0.0125 M substrate solution and 0.55 ml H_2O, and having a temperature of 40°.

Samples (0.1 ml) of the reaction mixture were taken at intervals and were pipetted into 2.0 ml of 0.2 M sodium citrate buffer pH 5, deactivating the enzyme. Ninhydrin solution (1.0 ml) was added and the amount of ninhydrin-positive material determined. Leucine was used as a standard. The colour value of the substrate (V_S) was calculated according to Moore and Stein [18] by extrapolating the colour to zero time.* The colour values of the products (V_I and V_{II}) were known. From the colour yield at zero time (E_0) and after 20 min (E_{20}) and the above colour values, the degree of hydrolysis (x %) could be obtained from

$$\frac{(100 - x)\,E_0}{100} + \frac{xE_0(V_I + V_{II})}{100\quad V_S} = E_{20}$$

From the value of (x), the relative rates were re-calculated ** to give % hydrolysis obtainable by using 0.1 µl enzyme solution per 2.5 ml reaction mixture having 0.005 M substrate solution (Table 1).

RESULTS AND DISCUSSION

The relative rates, given as % hydrolysis in 20 min of 2.5 ml 0.005 M substrate solution by 0.1 µl enzyme solution (activity $C_1 = 22$) are found in Table 1 and compared in Table 2. Phosphate-free peptides (Nos. 1 — 15), O-phosphorylated (16 — 26), O-monophenyl-phosphorylated (27, 28), and O-pyrophosphorylated serine peptides were used as substrates. It can be seen, that the enzyme hydrolyses dipeptides having N-terminal O-phosphorylserine (Fig. 1 a) about 250 times slower than the corresponding un-phosphorylated peptides. Dipeptides, having O-phosphorylserine in C-terminal position (*i.e.* the phosphate group being more distant from the free amino group, (Fig. 1 b) are hydrolysed less than 100 times slower than the corresponding serine peptides with a free hydroxyl group. If the phosphate group is placed far from the free amino group (Fig. 1 c) as in the tripeptide (No. 16) L-leucyl-glycyl-(O-phosphoryl)-L-serine, the rate of hydrolysis is about the same as that of L-leucylglycyl-L-serine (No. 3). The hydrolysis rate lowering effect given by

* The ninhydrin colour values of Ile-SerP (9 % of that of L-leucine) and of Ile-Ser (22 %) deviated considerably from those of the other compounds.

** The substrates were run at more than one enzyme concentration. Linearity between initial rate and enzyme concentration was found in all cases where significant hydrolysis was observed.

Table 1. Relative rates of hydrolysis by leucine-aminopeptidase, given as % hydrolysis in 20 min obtainable by using 0.1 µl enzyme solution (activity $C_1 = 22$; 0.88 mg N/ml) for 2.5 ml of 0.005 M substrate (*cf.* Experimental).

	Substrate	Actual amount enzyme used µl	% Hydrolysis in 20 min using 0.1 µl enzyme
1	L-Leu-Gly	0.1	20.0
2	L-Leu-L-Ser	0.2	10.0
3	L-Leu-Gly-L-Ser	0.25	13.9
4	L-Ser-L-Leu	0.3	8.3
5	L-Ile-L-Ser	1.5	1.6
6	Gly-L-Leu	2.5	1.3
7	α-L-Glu-L-Ser-L-Ala	1	1.1
8	L-Lys-L-Ser	1.5	0.78
9	L-Ser-Gly	5	0.37
10	DL-Ser-Gly	10	0.24
11	L-Ser-L-Ala	5	0.23
12	Gly-DL-Ser	50	0.065
13	α-L-Glu-L-Ser	10	0.052
14	Gly-Gly	50	0.031
15	γ-L-Glu-L-Ser	10	< 0.01
16	L-Leu-Gly-SerP	0.25	7.4
17	L-Leu-SerP	10	0.39
18	L-SerP-L-Leu	100	0.033
19	L-Ile-L-SerP	100	0.021
20	L-Lys-L-SerP	50	0.014
21	L-SerP-L-Ala	100	< 0.004
22	DL-SerP-Gly	100	< 0.001
23	Gly-DL-SerP	100	< 0.005
24	α-L-Glu-L-SerP	100	< 0.001
25	L-SerP-L-SerP	100	< 0.002
26	γ-L-Glu-L-SerP	100	< 0.001
27	DL-Serφ P-Gly	5	0.58
28	Gly-DL-Serφ P	50	0.04
29	L-Leu-L-SerPP	100	0.055
30	L-SerPP-L-Leu	100	< 0.003

a phosphate group is, therefore, directly proportional to its distance to the free α-amino-end.

A pyrophosphoryl group (Fig. 1 d), present in the substrates 29 and 30, seems to retard hydrolysis even more than a simple phosphate group (*cf.* corresponding substrates 2, 17 and 4, 18). On the other hand, the attachment of a hydrophobic benzene ring to the phosphate group (*cf.* Fig. 1 e) as in the substrates (27, 28) increases the rates of hydrolysis to the same or even higher value than that of the un-phosphorylated analogues.

Smith and collaborators have advanced a hypothesis for the action mechanism of leucine aminopeptidase. According to this hypothesis, substrate like L-leucylglycine is bound to the enzyme by »hydrophobic bonds» between the aliphatic side chain of leucine and a specific »hydrophobic site» in the enzyme.

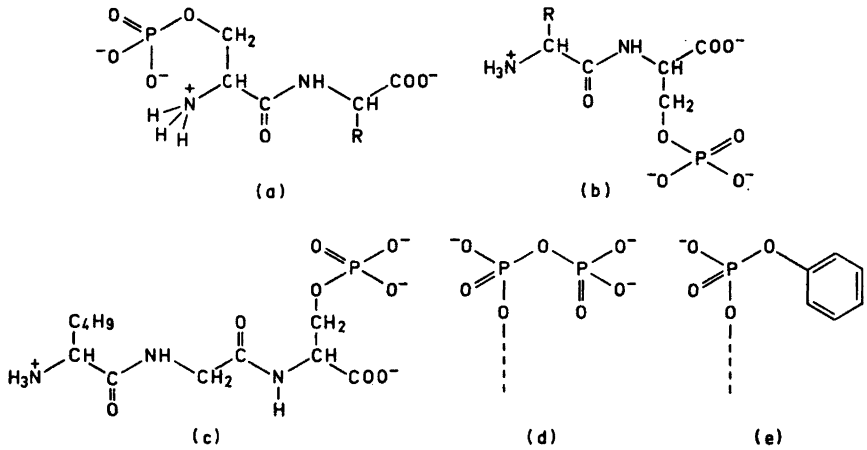

Fig. 1. (a) N-Terminal O-phosphorylserine dipeptide;
(b) C- » » » ;
(c) Leucylglycyl-(O-phosphoryl)-serine;
(d) O-Pyrophosphoryl group;
(e) O-Monophenylphosphoryl group.

The free substrate amino group and the peptide bond oxygen * atom thereby come into a favourable position to coordinate with the enzyme-bound metal (Mg^{2+} or Mn^{2+}) atom (Fig. 2 a). The metal atom increases the polarization

Table 2. Times decrease in the rate of leucine-aminopeptidase hydrolysis of serine peptides caused by phosphorylation, pyrophosphorylation or monophenylphosphorylation of the serine β-hydroxyl group.

Substrate	Times decrease in rate as compared with the corresponding phosphate-free peptide
SerPP-Leu	2500
Leu-SerPP	300
SerP-Gly	> 250
SerP-Ala	> 60
SerP-Leu	250
Gly-SerP	> 13
Leu-SerP	37
Ile-SerP	76
Lys-SerP	56
α-Glu-SerP	> 52
Leu-Gly-SerP	2
Gly-Serφ P	0
Serφ P-Gly	−2

* Smith orginally suggested metal coordination to the amide-nitrogen atom in the peptide bond. Later work has, however, shown, that the carbonyl oxygen atom is the more probable ligand.[19]

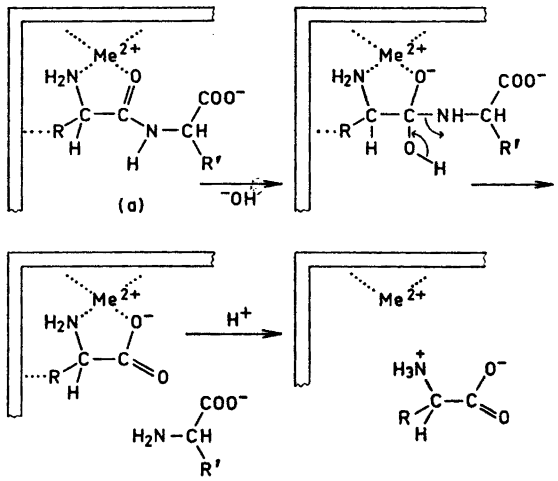

Fig. 2. Peptide bond hydrolysis by leucine-aminopeptidase, according to hypotheses of Smith *et al.* and of Rabin. The substrate is bound to a hydrophobic site and to the metal ion in the enzyme-substrate complex (a).

of the carbonyl group and, therefore, facilitates nucleophilic attack at the carbonyl carbon atom by water or hydroxide ion. Such an attack would repulse the amide nitrogen together with the peptide bond electrons, giving, after hydrogen transfer from water (Fig. 2) glycine. The final step would be dissociation of leucine from the »active site» because of protonation. The amino group of α-amino acids binds protons notably stronger than their amides or peptides (pK_S 7—8).

As pointed out by Malmström and Rosenberg,[20] it is possible, that the metal ion participates in a way different to that in the above mechanism. In fact, little is known about the mechanism of leucine aminopeptidase hydrolysis, as compared with, *e.g.*, chymotrypsin [21] or alkaline phosphatase.[22] Some remarks on the results obtained here could, however, be made, with the hypothesis of Smith *et al.* in mind. The process could be described as:

(1) metal activation of enzyme $\quad E + M \rightleftharpoons EM$

(2) enzyme-substrate complex formation $\quad EM + S \rightleftharpoons EMS$

(3) hydrolysis $\quad EMS + H_2O \rightleftharpoons EM + P_1 + P_2$

where E = enzyme, M = metal ion, S = substrate and P_1 and P_2 the products formed by hydrolysis of the peptide bond.

The equilibrium according to (1) is assumed to go far to the right, before the addition of substrate, as optimal [8] metal ion concentration was used (0.0022 M Mn^{2+}). By substrate addition, a competition between enzyme and substrate is possible:

(4) $EM + S \rightleftharpoons E + MS$

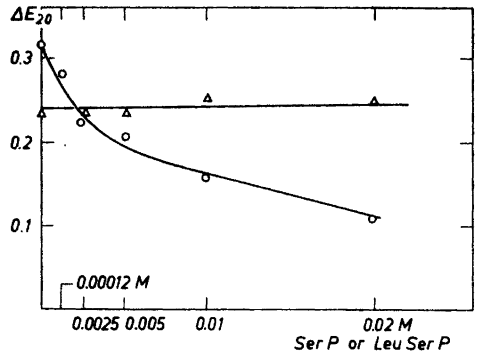

Fig. 3. Leucine aminopeptidase hydrolysis of L-leucyl-L-serine in the presence of different concentrations of O-phosphoryl-L-serine (△) or L-leucyl-(O-phosphoryl)-L-serine (○). Hydrolysis rates given as increase in optical density at 570 mμ (ninhydrin colour reaction) after 20 min reaction time (ΔE_{20}).

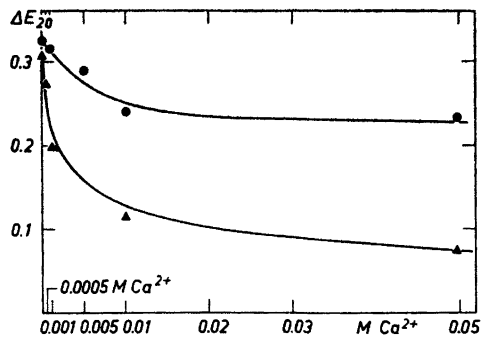

Fig. 4. Leucine aminopeptidase hydrolysis of L-leucyl-L-serine (▲) and L-leucyl-(O-phosphoryl)-L-serine (●) in the presence of calcium. Hydrolysis rates as above (Fig. 3).

Such competition seems, at first, possible in the case of O-phosphorylated peptides, binding Mn^{2+} considerably stronger than un-phosphorylated peptides.[23] However, the following reasons rule out such an explanation of the slow hydrolysis of the O-phosphorylated peptides:

a) The rates of hydrolysis of SerP-Leu, Leu-SerP and Leu-Gly-SerP differ greatly (Table 1), whereas rather small differences can be expected in their Mn^{2+} complex stability constants; in fact, the tripeptide could be expected to bind the metal most strongly.[23]

b) O-Phosphorylated peptides seem to inhibit moderately the hydrolysis of un-phosphorylated ones,* but O-phosphorylserine, being the strongest binding ligand [23] does not; (Fig. 3).

The explanation for the slow hydrolysis of the phosphopeptides by leucine aminopeptidase seems, therefore, to be found in a slow or unfavourable enzyme substrate complex formation according to (2) and/or slow hydrolysis of this complex (3).

Regarding eqn. (3), several reasons for slow hydrolysis seem possible: A phosphate group in the EMS complex might contribute in the coordination of the metal ion and thereby decrease the polarizing effect of the latter on the peptide carbonyl group (Fig. 5 a). The strongly negatively charged and solvated phosphate group should, also, hinder the attack by water or hydroxide ion on the carbonyl carbon. The probability of »wrong» coordination as well as the steric and electrostatic effects seems higher, the closer the amino and phosphate groups are situated.

* We tried to eliminate this inhibition by adding Ca^{2+} for complexing the O-phosphorylated peptide, as Smith *et al.* had reported,[8] that Ca^{2+} did not affect hydrolysis with leucine aminopeptidase. In the case of our enzyme preparation, however, Ca^{2+} inhibited hydrolysis of Leu-Ser as well as of Leu-SerP (Fig. 4).

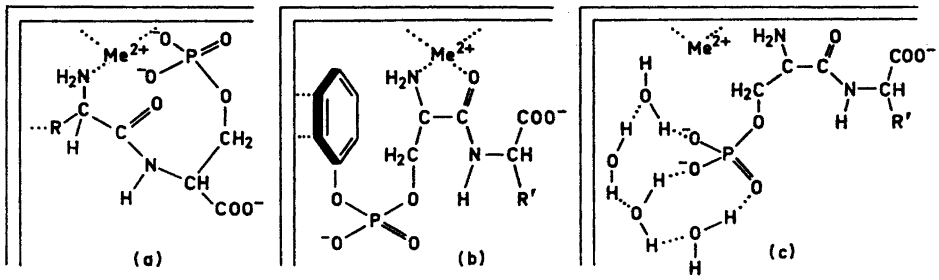

Fig. 5. O-Phosphorylated peptides as substrates for leucine aminopeptidase:

(a) A phosphate group may coordinate with the metal ion, suggested by Smith *et al.* to polarize the peptide bond carbonyl group.
(b) O-Monophenylphosphorylated peptides are good substrates, possibly because of affinity for a hydrophobic region of the enzyme.
(c) The hydration shell of a phosphate group may have to be broken, *i.e.* require energy, to enable normal enzyme-substrate complex formation.

Regarding eqn. (2) it is well known, that the enzyme prefers amino acids with hydrophobic side chains, such as leucine. The phosphate group is, however, one of the most hydrophilic groups known. Little attraction for O-phosphorylated peptides would be expected from a »hydrophobic site» in the active site of the enzyme, and the less, the closer the α-amino and phosphate groups are situated together in the peptide. This is in agreement with the hydrolysis rates found: N-terminal O-phosphoryl-serine dipeptides are very slowly hydrolyzed; C-terminal such ones faster, whereas the tripeptide, Leu-Gly-SerP, having the hydrophilic, hydrated phosphate group farther from the free α-amino end is hydrolyzed at about the same rate as Leu-Gly-Ser. These effects of hydrophobic and hydrophilic groups near the peptide bond hydrolyzed is further demonstrated by the following results: the monophenylphosphoryl derivatives (substrates 27 and 28; *cf.* Fig. 5 b), in spite of the negative charges on their phosphate groups, are hydrolyzed with the same rate or faster than the phosphate-free analogues, whereas the pyrophosphates (substrates 29 and 30), supposed to be hydrated even more than the monophosphate analogues (substrates 17 and 18), belong to the most resistant substrates.

A requirement for binding according to eqn. (2) and Fig. 2 is an uncharged free α-aminogroup. O-Phosphorylated peptides have their $pK_{NH_3^+}$ somewhat higher than the corresponding phosphate-free peptides[6,24] and their α-amino groups are therefore only about half-neutralized at the pH used for hydrolysis. We found, however, that the rate of hydrolysis of, *e.g.*, Ile-SerP was practically constant within the interval pH 7.5 — 9.5.

A biological consequence of the resistance of the phosphopeptides to the proteolytic enzymes is that they may be absorbed as such from the intestines and exist in organisms, intra- as well as extracellularily, and that they may have metal ion transport functions as proposed.[25] It is then, however, necessary that they are not exposed to phosphatases. We have demonstrated that O-phosphorylated serine peptides are hydrolysed by alkaline phosphatases

present in the intestine,[26] kidney, bone, and yeast.[27] A specific »phosphoserine phosphatase» is present in liver.[28] It is known that the phosphate in phosphoproteins has a high turnover rate and specific phosphoprotein phosphatases have been isolated.[29]

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