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Enzymic Degradation of a Phosphopeptide Obtained by Trypsin Hydrolysis of α-Casein. A Partial Structural Formula *

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A tryptic α-casein phosphopeptide has been subjected to the action of pepsin, leucine aminopeptidase and carboxypeptidases. The pepsin hydrolysates are fractionated on Dowex 1-X2 columns (magnesium acetate gradient). Paper electrophoresis, amino-terminal group analysis and quantitative amino acid determination on resulting peptide fractions indicate that pepsin splits the phosphopeptide into four main peptide fragments. Partial sequences of these peptides are obtained, so that they can be placed in unique positions in the molecule.

The seven phosphoamino acid residues are localized in the amino-terminal half of the peptide. Four of these can be arranged consecutively, while the others seem to be linked to different amino acid residues.

In previous communications [1,2] the isolation of a phosphopeptide from a tryptic hydrolysate of bovine α-casein was described. The composition of this peptide was determined,[2,3] and by titration its seven phosphate groups were found to be O-monoesterified to serine and threonine residues.[3]

The next stage in the study of the phosphopeptide has been to investigate the amino acid sequence. The information required to deduce a partial structural formula has been furnished by experiments made in this work.

EXPERIMENTAL

Preparation of the tryptic phosphopeptide. Two preparations were used. One (A) was identical to preparation II decsribed in Ref.[3] The other (B) was prepared in the same manner [2] with the exception of the chromatographic separation, which was modified to involve one run only by prolonged elution with 0.22 M magnesium acetate. The phosphopeptide, retained on the column, was found to be free from most of the other material.

Abbreviations: DNP, 2,4-dinitrophenyl; SerP, O-phosphorylserine; ThrP, O-phosphorylthreonine.

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Barium phosphopeptide (5.6 g; N 9.4; P 2.9; *cf.* Ref.[3]), dissolved in 0.3 M HAc, was applied to a Dowex 1-X2 (acetate form, 200-400 mesh, 2.8 × 60 cm) column. It was first eluted with 750 ml 0.3 M acetic acid, then with 600 ml 0.22 M magnesium acetate and finally with magnesium acetate of gradually increasing concentration. The mixing chamber (volume 1 l), containing 0.22 M magnesium acetate, was connected to an upper chamber filled with 1 M magnesium acetate. When 300 ml of this gradient solution had passed the column, the peptide came out, as indicated by ninhydrin after alkaline hydrolysis.[4] The 270—330 ml effluent was combined and the peptide precipitated in the magnesium form by acetone. It was dried with acetone, ether, and *in vacuo* at 45°; weight: 1.1 g (N 6.1; P 2.3); 15.4 % recovery of total α-casein P (*cf.* Ref.[2]). The identity (*cf.* Refs.[2,3]) was stressed by N/P atomic ratio (5.9), paper electrophoresis in 0.4 M HAc, 10 V/cm, 5 h (a single band), amino-terminal group analysis, quantitative amino acid determination and carboxypeptidase digestion (Tables 1 and 3).

Hydrolysis by pepsin. A 2 mM solution (~1 %) of the tryptic phosphopeptide* (preparation B) was digested with pepsin (twice crystallized, Armour Lot No. 71122) at 25°; enzyme/substrate ratios (by weight) 1/50 (pH 2.1) and 1/5 (pH 1.9 in citrate buffer.[5]) The course of hydrolysis was followed on 50 µl samples by the ninhydrin procedure.[6] The enzyme action was terminated after 24 h by increasing the pH to about 8, and the digests were freeze-dried immediately. Total increase in ninhydrin color was 3.5 and 4.5 times the initial values at the lower and higher pepsin concentrations, respectively.

Hydrolysis by carboxypeptidases. Carboxypeptidase A (Worthington Biochemical Corporation's recrystallized and diisopropylfluorophosphate-treated product, Lot No. 6111, 30 mg protein per ml) was immediately before use washed three times with ice water by centrifugation and then suspended in H_2O to the initial concentration. The incubation mixture contained 0.2 to 0.7 µmole peptide in 100 µl H_2O adjusted to pH 8 and 50 µl of carboxypeptidase A suspension.[7] This mixture was incubated at 40° for 18 h, and then kept frozen (−20°) until required.

A solution of carboxypeptidase B (8.4 mg N per ml) purchased from Worthington Biochemical Corporation (Lot No. 6045, prepared according to Folk *et al.*[8]) was dialyzed against 0.02 M phosphate buffer pH 7.65 for 2 h at 4°. The incubation mixture consisted of 0.3 µmole peptide in 50 µl H_2O adjusted to pH 7.7, 50 µl 0.02 M phosphate buffer and 10 µl dialyzed carboxypeptidase B solution. After incubation at 25° aliquots of 50 µl were removed at indicated times (Table 3); the reaction was stopped with 20 µl 0.2 M HCl, and the solution stored at −20°.

Hydrolysis by leucine aminopeptidase. The enzyme solution (0.88 mg N per ml, C_1 = 20) was a gift from Mr. L. Strid of this institute. It had been prepared according to Hill *et al.*[9] omitting the zone electrophoretic step. Final purification was made by chromatography on DEAE-cellulose.[10] The digestion mixture contained 0.3 to 0.7 µmole peptide in 70 µl H_2O adjusted to pH 8.5, 10 µl 0.05 M $MgCl_2$, 10 µl 0.5 M Tris-HCl buffer pH 8.5 and 10 µl leucine aminopeptidase solution. For peptide preparations, which contained magnesium ions, the incubation mixture was diluted to a final magnesium ion concentration of approximately 0.005 M (Ref.[11]). After incubation (*cf.* Tables 2 and 3) the solutions were stored at −20°. To obtain the same volume of the digests before analysis (see below) the dilute solutions were taken to dryness over KOH *in vacuo*, and the residues were dissolved in 100 µl H_2O.

Apart from the incubation mixtures mentioned each enzyme and peptide was incubated separately as blanks under identical conditions. No detectable amounts of amino acids could be found. It should also be emphasized that for the carboxypeptidase A and leucine aminopeptidase digests the conditions were chosen to yield maximal rather than limited digestions.

Chromatography of the peptic hydrolysates on Dowex 1-X2. Hydrolysates of 10 to 20 µmoles magnesium phosphopeptide, dissolved in 0.04 M HAc, were applied on Dowex 1-X2 (200—400 mesh, acetate form, 1.5 × 50 cm). Elution was made stepwise with 0.04 M HAc, and 0.4 M HAc, and then with magnesium acetate (pH~9) of gradually increasing concentration. The mixing chamber (volume 1 l), initially containing 0.1 M magnesium acetate, was connected to an upper chamber filled with 1 M magnesium

* A molecular weight 4415 was assumed.[3] Those assumed for the peptic peptides are based on the analyses listed in Table 1.

acetate. The flow rate was about 25 ml/h. Fractions of 8 ml were collected and 0.5 ml aliquots analysed by ninhydrin, directly [6] and after alkaline hydrolysis.[4]

Fractions under each peak were pooled and freeze-dried. Fractions containing magnesium acetate were desalted on a Dowex 50-X12 column (50 mesh, 3 × 12 cm, H^+-form) developed with water. Resulting ninhydrin positive effluent was freed from acetic acid by freeze-drying, dissolved in a small volume water, neutralized with dilute KOH, and freeze-dried again.

Paper electrophoresis. About 0.2 μmole peptide was applied on Whatman No. 1 paper. The runs were carried out at 21° and 10 V/cm in an LKB apparatus. A 0.1 M sodium acetate buffer, pH 3.7, gave the best separation of pepsin hydrolysates (*cf.* Ref.[12]). The buffers tested were 0.4 M HAc pH 2.6; 0.05 M sodium acetate buffer pH 5.0; 0.03 M sodium phosphate buffer pH 7.6. Buffers with pH < 2 (*e.g.* 20 % formic acid [13]) were not suitable due to low solubility of some of the present fractions. The papers were developed with 0.3 % ninhydrin in ethanol/acetic acid (99/1, by volume).

Amino-terminal determination. The peptide fractions (0.2 to 0.5 μmole) were reacted at 40° and pH 8.3 ($NaHCO_3$) with dinitrofluorobenzene in aqueous ethanol for 4 h.[14] DNP-peptides, *2-7* and *I-VII*, were hydrolysed with 6 M redistilled HCl in evacuated sealed tubes at 105° for 8 and 18 h, respectively. Fractions containing glycine were also hydrolysed for 4 h. DNP-amino acids were identified by paper chromatography in the *tert*-amylalcohol/phthalate buffer (pH 5.3) solvent.[15] At this pH both aspartic acid-glutamic acid and valine-isoleucine can be separated. The identification was sometimes checked by two-dimensional chromatography. The solvent mentioned was used in the first direction and 1.5 M phosphate buffer, pH 6.0, in the second direction.[1]

Quantitative amino acid analysis. Samples were hydrolysed with redistilled 6 M HCl in evacuated sealed tubes at 110° (Table 1). The analyses were performed according to Moore *et al.*[16] When a small amount of material was available, this method was used for acidic and neutral amino acids, and the analysis was completed with the dinitrophenylation method described by Koch and Weidel.[17] The solvents mentioned were used at the two-dimensional paper chromatography.

Identification of amino acids from exopeptidase digests. Aliquots of 20 to 50 μl digestion solution were applied on a Whatman No. 1 paper and subjected to paper chromatography in the solvents butanol/acetic acid/water (4/1/5, by volume, upper phase), descending, and phenol/water (4/1, by volume), ascending. The papers were developed with ninhydrin (0.1 %) and phosphate (ammonium molybdate) reagents. The intensity of the spots was rated from +1 to +4 by comparing with standard amounts of known amino acids treated in the same way.

Methionine was identified after treatment of the sample with 30 % H_2O_2 and 0.05 % ammonium molybdate.[18] The "methionine-valine" spot then completely disappeared. Instead two new spots appeared corresponding to methionine sulphone and methionine sulfoxide.

The presence of glutamine was checked by paper chromatography of its derivative, DNP-glutamic acid. The *tert*-amylalcohol and phosphate buffer solvents were used. The digest was treated with dinitrofluorobenzene, the DNP-glutamine separated by ether extraction, and the amide bond split by hydrolysis with 6 M redistilled HCl at 105° for 4 h.

The existence of O-phosphorylserine was confirmed by paper electrophoresis in 1 M HAc, 8 h and 10 V/cm. A guide-strip was cut out, and developed with ninhydrin and phosphate reagents. Material moving as synthetic phosphorylserine* was eluted with water and dephosphorylated by hydrolysis with 6 M redistilled HCl at 110° for 20 h. Serine was identified by paper chromatography.

RESULTS

Fig. 1 shows the chromatographic separation of the peptic split products obtained from the phosphopeptide at the different enzyme concentrations. The two curves (Fig. 1a,b) differ mainly in the front region, which may partly be caused by the high concentrations of pepsin and salt (sodium citrate)

* The O-phosphorylserine used was identical to that of a previous work.[19]

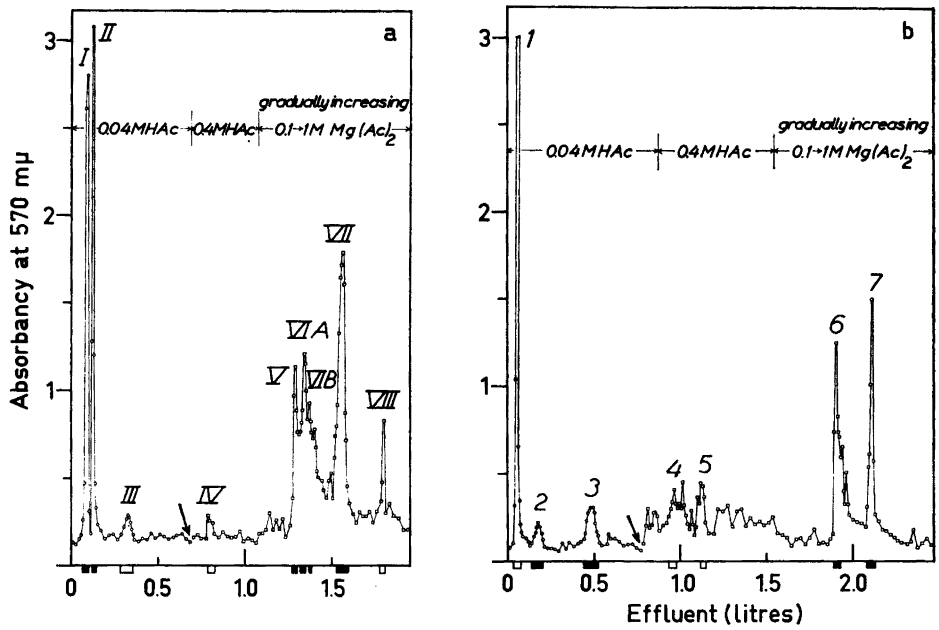

Fig. 1. Chromatography on Dowex 1-X2 columns (1.5 × 50 cm) of pepsin hydrolysates of the tryptic α-casein phosphopeptide. Ninhydrin analyses of effluent fractions were performed directly [6] (○) or after alkaline hydrolysis [4] (□; from the arrow). Between the peaks only every third analysis has been plotted. The *solid bars* indicate the fractions pooled, from which the peptides described in this paper were prepared.

a. Hydrolysate of 20 μmoles phosphopeptide obtained at the enzyme/substrate ratio 1/50.

b. Hydrolysate of 10 μmoles phosphopeptide obtained at the enzyme/substrate ratio 1/5.

present in one sample (Fig. 1b). Exploratory runs showed no further ninhydrin positive peak to appear, when the magnesium acetate concentration finally was increased to 2 M. Pooled fractions (Fig. 1a), indicated by black bars in the figure, represent 60 % nitrogen recovered (uncorrected for losses during preparation).

Fig. 2 shows the results of paper electrophoresis of the peptic peptides. Table 1 lists the amino acid composition of the peptides and their amino-terminal groups, as well as N/P atomic ratios. The destruction of serine was assumed to follow a first order reaction,[20] which gives 6 (5.7) residues of serine in the original peptide.[2] As a first approximation the same destruction figures were assumed for the peptic peptides, *i.e.* 60 % serine recovered after 72 h of hydrolysis (cf. Refs.[20,21]). Fractions *I*, *II*, *2* and *3* (Fig. 1) were contaminated with pepsin as concluded from the presence of phenylalanine and leucine, which are not found in the original phosphopeptide.[2] From the amount of leucine obtained, and the amino acid composition of pepsin,[22] values of analysis were corrected. Except for fraction *2* this correction never exceeded % 8 of an amino acid residue.

Table 1. Amino acid composition of tryptic *a*-casein phosphopeptide and of peptides obtained from it by pepsin digestion. The composition of each peptide is given as the molar ratios of the constituent amino acids. The values for the principle components are given in boldface type. The peptides are numbered as the corresponding chromatographic fractions (Fig. 1). The *P* number below each peptide designation represents the position of the peptide in the partial structural formula (Fig. 3). The other values within parentheses in the table head refer to time of hydrolysis. The serine figures of the peptic peptides have been corrected for destruction (*cf.* the text).

Amino acid	Tryptic peptide (prep. B)		Peptic peptides (72 h)									
			Enzyme/substrate = 1/50						Enzyme/substrate = 1/5			
	(22 h)	(72 h)	*I** (*P4*)	*II** (*P3*)	*V* (*P2*)	*VIA* (*P2*)	*VIB* (*P2*)	*VII* (*P1*)	*2** (*P4*)	*3** (*P3*)	*6* (*P2*)	*7* (*P1*)
Aspartic acid	4.00	3.84	**0.94**	0.26	**0.98**	**0.97**	**1.02**	**2.09**	**0.84**	0.04	**0.98**	**2.10**
Threonine	1.08	0.82	0.02	—	0.04	0.08	0.04	**0.88**	—	—	0.03	**0.85**
Serine	4.80	3.42	—	—	**3.88**	**3.61**	**4.08**	**1.83**	—	—	**4.10**	**2.20**
Glutamic acid	10.2	10.3	**2.23**	**1.51**	**4.13**	**4.45**	**4.32**	**2.97**	**2.27**	**1.01**	**4.17**	**2.85**
Proline	0.92	1.19	—	—	**1.11**	**1.00**	**1.01**	—	0.08	—	**1.01**	—
Glycine	0.92	0.96	0.03	—	0.13	0.04	0.05	**1.16**	—	0.03	—	**1.02**
Alanine	1.95	2.01	0.19	**0.97**	**0.93**	**0.90**	**0.77**	0.44	0.25	**1.00**	**0.95**	0.38
Valine	2.12	2.16	0.08	0.03	**1.84**	**1.92**	**1.83**	—	0.19	—	**1.88**	0.03
Methionine	1.73	1.91	**0.91**	**1.17**	—	0.10	0.08	0.09	**1.06**	**0.99**	0.03	0.04
Isoleucine	3.56	3.70	**1.22**	0.25	**1.84**	**1.79**	**1.73**	**0.97**	**1.06**	—	**1.61**	**1.02**
Lysine	1.93	2.08	**1.00**	0.2**	**0.94**	**0.90**	**1.0****	—	**0.9****	—	**1.0****	—
Total residues	35	35	6	3	16	16	16	10	6	3	16	10
Amino-terminal residue	Asp		Asp	Glu	Glu	Glu	Glu	Asp	Asp	Glu	Glu	Asp
N/P***	5.9		—	—	9.6	5.0	4.5	3.4	—	—	4.4	3.1

* Corrected for pepsin contamination based on the presence of 0.05 (*I*), 0.04 (*II*), 0.37 (*2*) and 0.04 (*3*) residues of leucine.[1]

** Obtained by Koch and Weidel's method.[17]

*** Calculated for *tryptic peptide* (41N/7P): 5.9; *P2* (18N/4P): 4.5; *P1* (10N/3P): 3.3; assuming 4; 1; 0; amide groups, respectively; see the text.

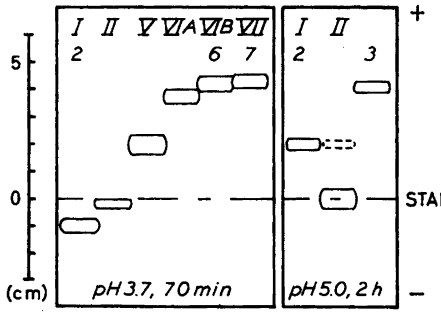

Fig. 2. Paper electrophoresis of chromatographic fractions (Fig. 1) from peptic hydrolysates of the tryptic α-casein phosphopeptide. Ninhydrin color, acetate buffers, 10 V/cm.

Peptide P1 [*Asp*(*SerP*,*Ileu*)-(*Asp*,*ThrP*,*Gly*,Glu_2)-(*SerP*,*Glu*)], *from studies on fractions VII and 7.** Carboxypeptidase A digestion (Table 2) indicates phosphorylserine and glutamic acid to be carboxyl-terminal amino acids. Dinitrophenylation gave DNP-aspartic acid (Table 1). Leucine aminopeptidase liberated aspartic acid, phosphorylserine and isoleucine (Table 2). In accord with the action of leucine aminopeptidase on synthetic phosphorylpeptides,[23] the good yield of phosphorylserine should suggest an amino-terminal sequence Asp-SerP-Ileu-... rather than Asp-Ileu-SerP-...

Peptide P2 [*Glu-Ala-SerP-SerP*($SerP_2$,$Ileu_2$,Glu_2,Val_2,*Asp*,*Lys*,*Pro*)-*GluN*H_2], *from studies on fractions V, VIA, VIB and 6.* These fractions have the same amino acid composition within an experimental uncertainty of ±5 % (Table 1), and they may thus come from the same amino acid sequence. They differ, however, in N/P ratio (Table 1). This might be explained by a more effective deamidation during the 1:5 pepsin hydrolysis (*6*) and a partial dephosphorylation during the 1:50 hydrolysis (*V*). The number of (amide; phosphate) groups: (2;2) *V*, (2;4) *VIA*, (1;4) *VI B* and *6* gives the N/P ratios 9.5, 4.8, 4.5, which may be compared to those observed (Table 1): 9.6, 5.0, 4.5 and 4.4, respectively.

Table 2. COOH-terminal and NH_2-terminal amino acids of peptic peptides.

Peptide		Enzyme	Conditions	Amino acids liberated
P1	*VII*	Carboxypeptidase A	40°, 18 h	Glu(+1); SerP(+1)
	7	Carboxypeptidase A	40°, ,,	Glu(+2); Serp(+1); Ala(+1)
	7	Leucine aminopeptidase	35°, ,,	Asp(+3); SerP(+3); Ileu(+3)
P2	*V***	Carboxypeptidase A	40°, 18 h	$GluNH_2$(+4)
	VIA	Carboxypeptidase A	40°, ,,	$GluNH_2$(+4)
	VIB	Carboxypeptidase A	40°, ,,	$GluNH_2$(+4)
	*6***	Carboxypeptidase A	40°, ,,	$GluNH_2$(+4); traces of Glu
	V	Leucine aminopeptidase	35°, ,,	Glu(+3); Ala(+3); Ser(+1); SerP(+1)
	VIA	Leucine aminopeptidase	35°, ,,	Glu(+2); Ala(+2)

* Unidentified glutaminyl residues are in all formulas represented as glutamyl (Glu) residues.
** Identical results were obtained by carboxypeptidase B at 25°, 18 h.

Dinitrophenylation gave DNP-glutamic acid (Table 1). Leucine aminopeptidase liberated glutamic acid, alanine, phosphorylserine and serine from fraction *V*, while it liberated glutamic acid and alanine from fraction *VIA* (Table 2). Since in this case partial dephoshorylation is required to release detectable amounts of phosphorylserine (serine),[23] two consecutive phosphorylserine residues are suggested. The action of carboxypeptidases A and B gave only glutamine (Table 2). We think that this is due to an adjacent proline residue and propose a carboxyl-terminal sequence ...-Pro-X-GluNH_2 or ...-Pro--GluNH_2.[24] Phosphorylserine in the same position as proline should have at least appeared as a trace in the hydrolysate of fraction *V*.

Peptide P3 [*GluNH_2(Ala,Met)*], *from studies on fractions II and 3.* Dinitrophenylation gave DNP-glutamic acid. The composition is given by the fraction *3* analysis (Table 1). Amino acid analysis of fraction *II* suggests a peptide Glu(Ala,Met) contaminated with about 20 % *P4* (see below). The main peptide of this fraction has zero net charge at pH 5, while peptide *3* has negative net charge (Fig. 2). The glutamyl group of peptide *II* may therefore be amide bound, but that of peptide *3* may not. We believe that the latter peptide has been produced by deamidation during hydrolysis (*cf.* under *P2*).

Peptide P4 [*Asp(Glu$_2$,Met,Ileu,Lys)*], *from studies on fractions I and 2.* Dinitrophenylation gave DNP-aspartic acid and traces of DNP-glutamic acid (Table 1). Though both fractions show some contamination, the results (Table 1, Fig. 2) are best explained with a peptide of the composition indicated. Further evidence for an amino acid sequence with this composition was obtained by carboxypeptidase B digestion of the tryptic phosphopeptide (see below).

Partial amino-terminal sequence [*Asp(SerP,Ileu)*-...]. Dinitrophenylation [1,2] (Table 1) and leucine aminopeptidase digestion of the original phosphopeptide (Table 3) gave identical results to those obtained by these methods on peptide *P1*. This peptide shall thus be amino-terminal.

Table 3. COOH-terminal and NH_2-terminal amino acids of the tryptic α-casein phosphopeptide.

Preparation	Enzyme	Conditions	Amino acids liberated
A	Carboxypeptidase B	25°, 30 min	Lys(+4); Ileu(+2); Met(+1); GluNH_2(+1); traces of Glu,Asp,Ala
A*	Carboxypeptidase B	25°, 2 h	Lys; Ileu; Met; GluNH_2; Glu; Asp; Ala; all these (+4)
B	Carboxypeptidase B	25°, 10 min	Lys(+4); Ileu(+1); traces of GluNH_2; Met
B*	Carboxypeptidase B	25°, 4 h	Lys; Ileu; Met; GluNH_2; Glu; Asp; Ala; all these (+4)
A	Leucine aminopeptidase	35°, 18 h	Asp(+3); SerP(+3); Ileu(+3)

* No additional amino acids were released after 18 h hydrolysis.

Partial carboxyl-terminal sequence [...-*GluNH*$_2$(*Ala,Met*)-*Asp-Glu*(*GluNH*$_2$, *Met*)-*Ileu-Lys*]. The specificity of trypsin suggests lysine to be the carboxyl-terminal amino acid residue.[1,2] This is confirmed by the carboxypeptidase experiments, which also give a partial sequence (Table 3). The release of methionine together with lysine from the original peptide uniquely shows peptide *P4* to be carboxyl-terminal. Since *P4* (fractions *1*, *2*) has negative net charge at pH 5.0 (Fig. 2); it contains only one residue glutamine and ...-Asp-Glu-... shall be a part of its sequence. Liberation of alanine indicates the subsequent peptide to be *P3* (Table 3). The peptide *P2* also contains alanine, but it is not split by carboxypeptidases (Table 2).

*Partial structural formula.*The four peptides, *P1-P4*, identified from the peptic hydrolysates account for the amino acid composition of the tryptic α-casein phosphopeptide (Table 1). Two of these peptides have been localized in the carboxyl-terminal part of the molecule, and one in the amino-terminal part. The unique positions of the peptic peptides are then known, and a partial structural formula could be deduced (Fig. 3).

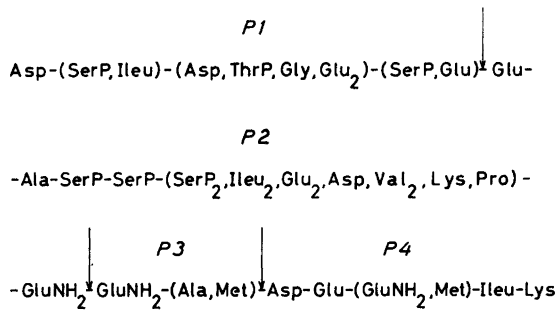

Fig. 3. Partial structural formula of the tryptic α-casein phosphopeptide. The arrows indicate major points of peptic hydrolysis. The peptides shown between these arrows are designated *P* numbers that correspond to the Fig. 1 fractions as indicated in Table 1.

As shown by this formula the positions of three peptide amide groups have been identified. The fourth may be found in the *P2* sequence (*cf.* N/P ratios of fractions *V* and *VIA* in Table 1).

DISCUSSION

The broad specificity of pepsin has been utilized in several studies to resolve amino acid sequences of peptides obtained from proteins by the action of trypsin.[25–27] In this work a tryptic α-casein phosphopeptide was split by pepsin into four main peptide fragments, which could be separated on Dowex 1-X2. Prior to analysis the purity of chromatographic fractions was checked by paper electrophoresis. No attempts were made to extend the separation procedure further, since dephosphorylation and other rearrangement reactions might increase.

The nitrogen recovery of the four peptic peptides (Table 1), calculated for fractions, *I, II, V-VIB, VII* of the Fig. 1a run, was 67 (*P4*), 48 (*P3*), 60 (*P2*) and 59 (*P1*) per cent, respectively. The occurrence of transpeptidation (see the review [28]) can thus be excluded.[4] These rather good yields and the fact that similar results are obtained with different pepsin concentrations indicate that no autodigestion products of pepsin are involved.

The exopeptidases used (Tables 2 and 3) do not function satisfactorily on peptide bonds, which involve phosphorylserine residues (*cf.* Refs.[23,29] and the review [30]). For carboxypeptidase A this might be due to the negatively charged phosphate group, since at the pH employed this enzyme also reacts slowly on acidic amino acid residues.[24,31] Leucine aminopeptidase on the other hand readily releases these amino acids from peptide chains.[32] Its decreased activity may therefore indicate the phosphate group to be more specifically involved, *e.g.* by combining with the activating metal ion.[32,33]

The nature of the phosphate bonds of the peptide was recently studied by titration.[3] All the phosphate groups were found to be O-monoesterified to serine and threonine residues. The existence of some phosphorylserine residues is now confirmed by their enzymic release (Tables 2 and 3). Phosphorylserine and phosphorylthreonine have previously been isolated from casein after hydrolysis with hydrochloric acid.[30] This treatment, however, does not exclude rearrangements of phosphate groups (N to O migration,[30,34] cleavage of diester and pyrophosphate bonds). Such reactions should be almost eliminated at enzymic hydrolysis, *e.g.* at hydrolysis by trypsin followed by aminopeptidase (Table 3).

Several phosphopeptides have been isolated from casein, whose compositions conform to the formula of Fig. 3; SerP-Glu (Refs.[21,35] *cf.* the review [30]), SerP-SerP (Ref.[21]), Glu-SerP (Refs.[21,36]) and Ala-SerP (Ref.[36]). In some of these studies [21,36] dilute hydrochloric acid at high temperature was used for hydrolysis, and a sequence inversion can have occurred.[12,37,38] This means that peptides reported as SerP-Ala and SerP-Asp (Ref.[21]) can have been reversed, and primarily thus been sequences in the present peptide. Using concentrated hydrochloric acid at 37° for hydrolysis Williams and Sanger [13] obtained whole casein peptides of the composition $(SerP)_n$, $n=1$, 2, 3; ($SerP_2$, Glu)*; ($SerP_3$,Glu,Leu)*. Due to these findings and to those reported in the literature [39–41] the authors [13] proposed that casein may contain local aggregations of phosphorylserine residues. Heald [42] investigated both whole casein and α-casein in similar manner and confirmed the existence of such peptides. However, he also found a small amount of a peptide fraction ($SerP_4$,Glu, Ala,Leu)*. The partial structural formula (Fig. 3) may account for these peptides, if leucine can be substituted for isoleucine in their compositions.

In accord with the suggestion of Williams and Sanger [13] the seven phosphoamino acids of our peptide appear to be concentrated within a peptide chain of only 15 to 20 residues (Fig. 3). However, maximally four can be arranged consecutively, while the others are apparently linked to different amino acid residues.

* With the exception of phosphorylserine the number of amino acid residues was not reported, since qualitative analysis had been performed.

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