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Chemical phosphorylation of bovine casein: relationships between the reacting mixture and the binding sites of the phosphoryl moiety

A. L. Medina,[a] D. Mesnier,[b] G. Tainturier[b] & D. Lorient[b]

[a]*Lab. FIRP Ingenieria Química and Dept. de Ciencia de los Alimentos, Facultad de Farmacia, Universidad de los Andes, Mérida, Venezuela*

[b]*Département de Biochimie et Toxicologie Alimentaires ENS, BANA Campus Universitaire 21000, Dijon, France*

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The aim of this work was to determine the influence of pH on the chemical phosphorylation of bovine casein by $POCl_3$. Experiments were performed at pH 5, pH 7 and pH 9. A pH-stat apparatus was used to maintain the pH at a fixed value. The phosphorus content of modified casein was measured by the Fiske and Subbarow method. The amount of unreacted ϵ-amino groups of lysyl residues was determined using 2,4,6-trinitrobenzene sulfonic acid.

Polyacrylamide gel electrophoresis in a dissociating medium showed that many of the modified species were linked together by stable bonds. This observation is consistent with the very high viscosity of the product in dissociating medium. An amino acid analysis was performed to check the possible production of a lysino-alanine linkage.

The stability of the modified casein was investigated by partial hydrolysis at pH 2, pH 5 and pH 10.5, followed by polyacrylamide gel electrophoresis.

The results showed that the number and nature of phosphate bonds depend on the pH at which the reaction was carried out. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The chemical phosphorylation of proteins sometimes leads to controversial results. This reaction is difficult to control and, as well as the nature of the investigated protein, many other parameters probably account for the different results obtained by different workers. Some studies show that the phosphoryl moiety, chemically linked to proteins, is bound either to the oxygen of hydroxylated amino acids (Ullman & Perlman, 1975; Willmitzer & Wagner, 1975) or to the nitrogen atom of amino or imidazole groups (Heidelberger *et al.*, 1941; Meyer & Heidelberger, 1946; Woo *et al.*, 1982). Matheis *et al.* (1983) found that, for casein, phosphate binds only to hydroxyl groups of amino acids such as serine, threonine or tyrosine (Salak *et al.*, 1965), while lysozyme is almost solely phosphorylated on the nitrogen atom of free amino groups.

Working on whole casein, we obtained different results, and in some cases we found evidence of nitrogen–phosphorus bonds. In the present study, we carried out phosphorylation with $POCl_3$ (Matheis & Whitaker, 1984); the size of the resulting molecules was determined and the types of bonds involved as well as their stability were investigated.

MATERIALS AND METHODS

Preparation of casein

Bovine casein was obtained from skimmed milk by isoelectric precipitation at pH 4.6 with 1 M HCl solution. The precipitate was washed and resolubilized at pH 7 in NaOH solution. The casein was reprecipitated and washed three times.

Following the last wash, the casein was resolubilized at pH 7, freeze-dried and preserved in a hermetically sealed bag at 4°C.

Phosphorylation

$POCl_3$ solution in CCl_4 (20/80, v/v) in a ratio of 1000 mol mol^{-1} casein was used.

The reaction of $POCl_3$ with water is exothermic and produces phosphoric and hydrochloric acids according to the overall equation:

$$POCl_3 + 3H_2O \rightarrow H_3PO_4 + 3HCl$$

The temperature was maintained at 4°C by an ice/water bath. The inorganic acids produced were neutralized *in*

situ with 5 M aqueous NaOH with the help of a pH-stat apparatus. After addition of the $POCl_3$ solution, the pH was maintained at a constant value for 60 min. Three different pH values were tested: 5, 7 and 9. The organic layer was removed and the mixture was allowed to settle for 12 h at 4°C before it was centrifuged (6000 *g*, 20 min, 25°C). The supernatants were dialysed against distilled water for 32 h while controlling the conductivity of the dialysate. Phosphorylated casein was freeze-dried and kept at 20°C in a hermetically sealed flask.

Phosphorus and nitrogen analysis

Phosphorus was determined by a modification of the method of Fiske & Subbarow (1925) as reported by Delsal & Manhouri (1958).

The mean molecular weight of casein is assessed to be 23 000 and an average of 6 mol of phosphorus mol^{-1} of casein is assumed to be naturally present. The total amount of phosphorus (total P) was measured after sulfoperchloric mineralization, and the inorganic-free phosphorus (free P) was determined without any mineralization. Calculations were performed in accordance with the following equation:

$$\text{Chemically bound P} = \text{(Total P)} - \text{(Natural P)} - \text{(Free P)}$$

Nitrogen was measured by the Kjeldahl method.

Titration of lysine residues

2,4,6-Trinitrobenzene sulfonic acid (TNBS) reacts specifically with primary amino groups (Habeed, 1966; Kakade & Liener, 1969; Fields, 1979). Trinitrophenylcasein is yellow and absorbs at 335 nm, and the higher the extent of the modification, the lower the absorption. The degree of modification is given by:

$$t(\%) = \frac{(OD)_{control} - (OD)_{modified}}{(OD)_{control}} \times 100$$

where $(OD)_{control}$ and $(OD)_{modified}$ represent the optical density measured for unmodified and modified casein solutions, respectively.

Lysinoalanine determination

To verify that the decrease in the number of primary amino groups did not originate from lysinoalanine formation, the modified protein was hydrolysed (110°C, 21 h, 6 M HCl) and the hydrolysate was quantified on a Beckman amino acid analyser (Model 119 CL) using the Moor & Stein (1963) method with a cation-exchange column (W3H). The amino acids were eluted by stepwise increase of the pH and ionic strength of the buffer and quantified by reaction with ninhydrin and absorption at 570 nm (and 440 nm for proline). Positive references containing lysinoalanine were obtained by heating an aliquot at pH 12 of both control and modified casein samples at 90°C for 1 h.

Electrophoresis

The samples were characterized by electrophoresis in a dissociating medium containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol, using two different techniques:

(1) Vertical polycrylamide gel electrophoresis using discontinuous buffers (Laemmli & Favre, 1973) in 15% acrylamide gel. The stacking gels were 4 cm high, the resolving gels 12 cm high, 1.5 mm thick and 12 cm wide. The separation was run with a constant intensity of 50 mA for two slabs over 6 h. The samples contained 0.5% protein and 5% 2-mercaptoethanol and were boiled for 5 min prior to deposition. Bromophenol blue was added as an indicator.

(2) Vertical polyacrylamide gel electrophoresis. This was performed by the method of Ouali (1983). A 3–10% linear gradient of acrylamide was used, suitable for the range of molecular weights (14 000–950 000) of the proteins to be separated in our samples.

Hydrolysis of phosphoryl groups

Hydrolyses were performed in acid or basic medium, at pH 2, pH 5 and pH 10.5. The pH of a 0.5% aqueous solution of phosphorylated casein was adjusted using 0.5 M HCl or NaOH solution and the resulting mixture was heated at 100°C for 10 min. The products were analysed by polyacrylamide gel electrophoresis containing 15% SDS and 5% 2-mercaptoethanol. Proteins were stained with Coomassie Brilliant Blue G.

RESULTS AND DISCUSSION

The pH of the reacting mixture influences the number and nature of phosphorylation sites. The control of the reaction parameters was sufficiently satisfactory to give reproducible results showing a dramatic effect of pH. The degree of phosphorylation is largely enhanced in neutral or alkaline medium as shown in Table 1, but the products also differ in the binding sites of the phosphorylated residues.

Table 1. Influence of pH on the overall degree of phosphorylation

	pH		
	5	7	9
Degree of phosphorylation (mol P mol^{-1} casein)	5.05 ± 0.35	11.43 ± 0.52	18.51 ± 0.67

The values indicated are averaged over seven independent experiments (method of Delsal & Manhouri, 1958).

Table 2. Determination of primary amino groups by the TNBS method

	pH	
	5	9
Percentage of lysyl residues reactive to TNBS	98.2	19.1
Number of lysyl residues blocked by phosphorus	0	15

TNBS, 2,4,6-trinitrobenzene sulfonic acid.

Results of TNBS determination of primary amino groups are presented in Table 2. The first column shows that, at pH 5, lysyl residues in phosphorylated casein are not blocked and consequently 98.2% of these residues are reactive to TNBS. On the other hand, at pH 9, 15 residues are blocked by phosphorus and therefore only 19.1% are reactive to TNBS.

The product of the reaction performed at pH 9 clearly shows a major modification of the ε-amino groups of lysyl residues. Since the formation of dehydroalanyl residues via 1,2-elimination of monohydrogen phosphate from phosphoseryl residues is a ubiquitous mechanism of degradation of phosphorylated proteins, the decrease of ε-primary amino groups by their 1,2-addition on dehydroalanyl residues could not be ruled out.

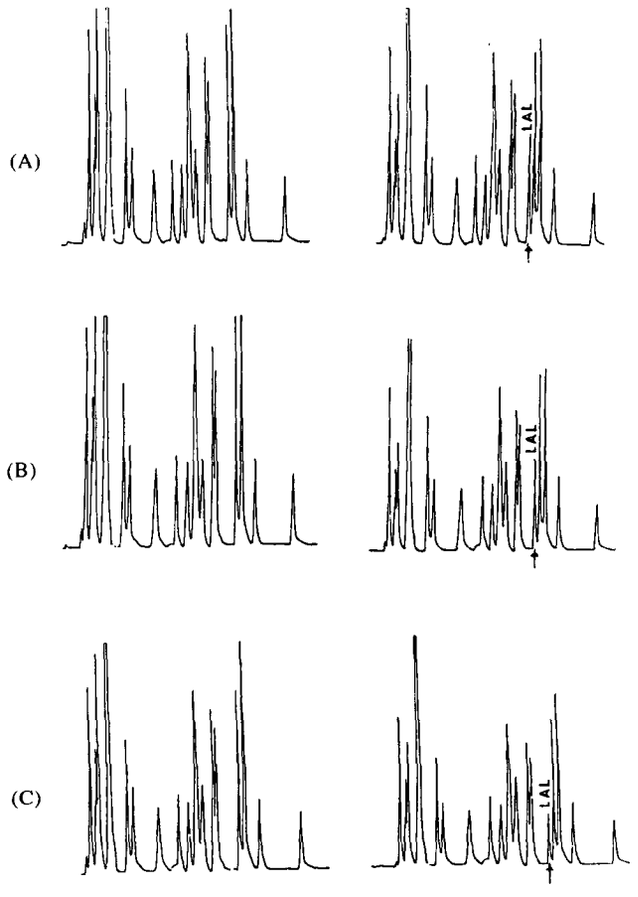

Fig. 1. Amino acid analysis of control casein (A) and casein phosphorylated at pH 5 (B) and pH 9 (C) before (left) and after (right) heating at 90°C, pH 12 for 1 h.

The formation and subsequent reaction of dehydroalanyl residues with lysyl amino groups should result in the cross-linking of protein chains, leading to an atypical amino acid such as lysinoalanine by complete hydrolysis of peptidic bonds.

The chromatograms of the hydrolysates relative to the three different phosphorylated caseins are shown in Fig. 1. None of them show a lysinoalanine peak, definitely ruling out the hypothesis of a decrease of lysyl amino groups by lysinoalanyl groups in the protein mixture.

The peak corresponding to lysinoalanine in the aminograms shown in Fig. 1 has been identified using an authentic sample. The same pattern is present in the aminogram on the right part of Fig. 1, which is relative to the same samples when heated at 90°C for 1 h in aqueous solution at pH 12 prior to hydrolysis. All the samples produce lysinoalanine upon hydrolysis, but the amount of this amino acid is higher in the samples resulting from phosphorylation at pH 5. This observation is consistent with the highest availability of unphosphorylated lysyl residues in the protein obtained at pH 5.

Stability of phosphate bonds

The *O*-phosphate bonds are acid-stable and base-labile. *N*-phosphate bonds are acid-labile and stable at high pH (Matheis *et al.*, 1983). In order to study the stability of phosphate bonds, hydrolysis was carried out at pH 2, pH 5 and pH 10.5, followed by polyacrylamide gel electrophoresis. The results are shown in Fig. 2. Casein phosphorylated at pH 9 is affected by hydrolysis at pH 2, even after a short treatment, although it is not modified after hydrolysis at either pH 5 or pH 10.5.

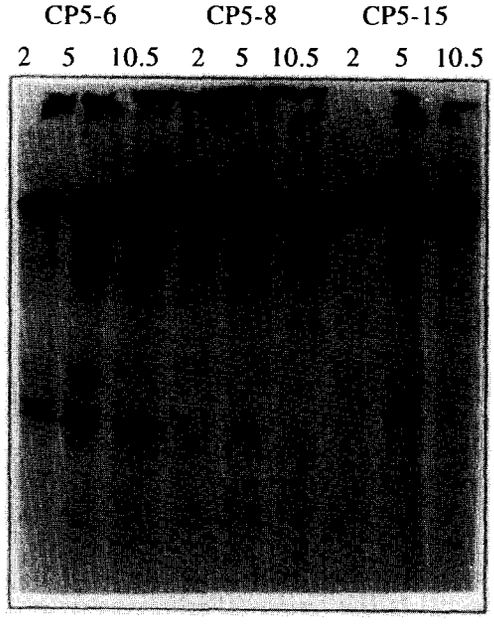

Fig. 2. SDS polyacrylamide gel electrophoresis of casein, phosphorylated at pH 5, pH 7 or pH 9, after hydrolysis at pH 10.5, pH 5 or pH 2 for 10 min. Sample aliquot: 20 μl of 0.5% protein solution.

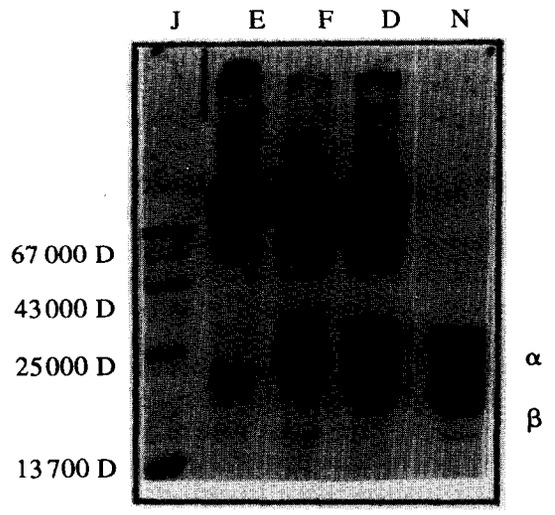

Fig. 3. SDS polyacrylamide gel electrophoresis of casein (N) and casein phosphorylated at pH 5 (D), pH 7 (E) and pH 9 (F). Reference proteins (J) from top to bottom: bovine serum albumin (BSA), MW 67000; ovalbumin, MW 43000; chymotrypsinogen A, MW 25000; ribonuclease, MW 13700 (with 2-mercaptoethanol). Sample aliquot: 20 μl. Proteins stained with Coomassie Brilliant Blue.

Casein phosphorylated at pH 5 and pH 7 does not show any modification after hydrolysis at pH 2, pH 5 or pH 10.5, even after 30 min.

After hydrolysis at pH 2, the phosphorylated casein at pH 9 can enter the gel and migrate, which is not possible before hydrolysis. This shows that, not only is the number of linked phosphate groups different, but also that the binding sites and the type of linkage differ.

Influence of phosphorylation on molecular size

To determine the polymer size, electrophoresis was performed in the presence of SDS and 2-mercaptoethanol (Fig. 3). Part of the sample remained at the bottom of the well in the stacking gel (particularly at pH 7). Another part of the protein mixture stopped at the beginning of the resolving gel (pH 5, pH 7 and pH 9). Bands corresponding to the native casein were present at pH 5. Between these bands and the well there was a trail produced by larger size molecules. The trail beyond the bands of non-phosphorylated casein is similar to those found with control casein.

The results of this experiment were confirmed by polycrylamide gel electrophoresis with a linear acrylamide gradient (from 3% to 10%) (Fig. 4). It can be seen that, with phosphorylated casein, a large part of the sample was retained in the upper part of the gel, whereas the fractions of non-phosphorylated casein migrated to the bottom.

These results confirm the observations of Woo *et al.* (1982) and Matheis *et al.* (1983). According to these authors, phosphorylation using $POCl_3$ causes intermolecular links by formation of *O,O'*-phosphodiester bridges and *N,N'*-phosphodiamide as well as isopeptidic links. To check that these links were not phosphocalcic

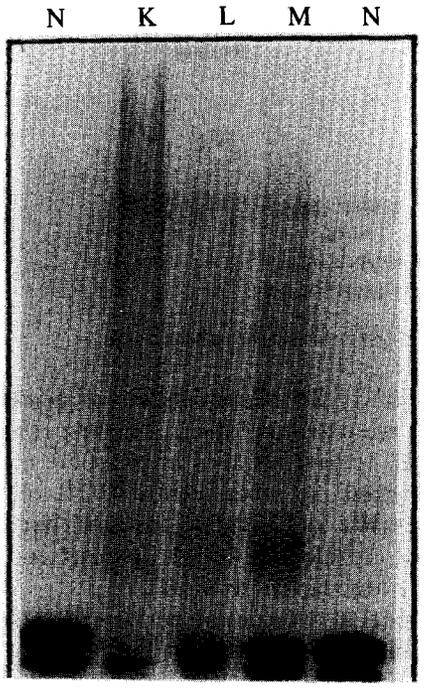

Fig. 4. SDS polyacrylamide gel electrophoresis of casein (N) and casein phosphorylated at pH 7 (K), pH 9 (L) and pH 5 (M); linear concentration gradient 3–10% in 2-mercaptoethanol. Sample aliquot: 20 μl. Proteins stained with Coomassie Brilliant Blue.

bridges, the samples were processed with EDTA, which chelates calcium ions. The results were unchanged.

CONCLUSION

The average amount of phosphorus fixed to bovine casein is highest when the reaction is carried out at neutral or alkaline pH.

Experimental evidence shows that, when the pH can be precisely controlled during the phosphorylation reaction, it is possible to predict the type of resulting product:

- At pH 5 or pH 7, most of the phosphate groups are bound on hydroxy amino acids of bovine casein.
- At pH 9, the rate of phosphorylation of bovine casein is highest due to the fact that both the hydroxyl groups of amino acids and the amino groups of lysyl residues are reactive.
- At pH 9, the phosphate groups combine with the bovine casein by acid-labile bonds (*N*-phosphate bonds).
- At pH 9, only 19% of lysyl residues are free and they are therefore able to form lysinoalanine residues.
- It is thus conjectured that, at pH 9, most of the phosphates are bound to the lysine of chemically phosphorylated casein.

Phosphorylation of casein creates intermolecular links, probably of the covalent type, and results in a population of particles with a size distribution.

The resulting phosphorylated molecules are bound to each other by stable links, which cannot be disrupted either by calcium chelating agents or by dissociating agents such as urea and mercaptoethanol.

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