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A KINETIC ANALYSIS OF THE DEPHOSPHORYLATION, BY BOVINE SPLEEN PHOSPHOPROTEIN PHOSPHATASE (EC 3.1.3.16) OF A PHOSPHOPEPTIDE DERIVED FROM β-CASEIN *

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Summary

A peptide containing the four closely grouped phosphoseryl residues present in β-casein has been enzymatically dephosphorylated with bovine spleen phosphoprotein phosphatase (EC 3.1.3.16). The course of the dephosphorylation reaction has been followed by cellulose acetate electrophoresis and the amount of partially phosphorylated peptides present at each stage quantified by the same method. The phosphate groups are shown to be removed in a sequential manner and the rate constants for each stage of the dephosphorylation have been computed from the data obtained. The rate constants indicate that interaction in the intact peptide results in an enhancement of the activity of the phosphoseryl cluster.

Introduction

Sequence studies have shown that the milk proteins α_{s1}-casein and β-casein both contain a number of phosphoseryl residues including in each protein a cluster of four grouped within a short length of the polypeptide chain [1—3]. Similar "phosphate centres" occur in chicken and fish phosvitins [4] and it has been suggested by a number of authors [5,6] that these sequences of phosphoseryl residues might be involved as active sites in cellular metabolism. If such "phosphate centres" are responsible for the ability of these proteins to participate in phosphate transfer reactions, it might be argued that, by analogy with ATP and other active phosphate compounds, one or more of the phosphoseryl residues should show an enhanced rate of hydrolysis.

This possibility has been investigated by studying the enzymic dephosphorylation of the "phosphate centre" in β-casein as an example of a phosphate

Abbrevations: P_4, P_3, P_2, P_1 for phosphopeptides with 4, 3, 2, 1 intact phosphoserine residues.

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transfer reaction. The sequence of four phosphoseryl residues in β-casein was chosen for this study because of the ready availability [3] of a peptide consisting of the first 25 residues of the protein chain and including the cluster of phosphoseryl residues -Ser*P*-Leu-Ser*P*-Ser*P*-Ser*P*-. The use of this peptide provides a simplified substrate for investigation of the dephosphorylation of the "phosphate centre" uncomplicated by the conformational effects that could occur with β-casein itself. It has been possible, using electrophoresis on cellulose acetate as the analytical method, to determine the rate at which the individual phosphoseryl residues are hydrolysed and to show that the first phosphoseryl residue is hydrolysed at a faster rate than the others.

The results of this investigation are reported in this communication.

Materials and Methods

Inorganic phosphate was measured by the method of Martin and Doty [7] modified by the exclusion of the isobutanol-benzene extraction step. The total phosphorus content of the peptides was determined by digestion with $HClO_4$ according to the procedure of Allen [8]. Protein was measured by the method of Lowry et al. [9].

Phosphoprotein phosphatase activity was measured during the enzyme purification in a test solution containing β-casein (1 mg), sodium acetate buffer, pH 5.8 (200 μmol), mercaptoethanol (2 μmol) and enzyme in a total volume of 1 ml. After incubation for 30 min at 37° C the reaction was stopped by the addition of silicotungstic acid (5.1% (w/v) $SiO_2 \cdot 12WO_3 \cdot 26H_2O$ in 0.75 M H_2SO_4, 500 μl). The precipitated peptide was removed by centrifugation and the amount of inorganic phosphate liberated was determined on an aliquot of the supernatant.

β-Casein was prepared from the skimmed milk of individual Ayrshire cows by acid precipitation followed by chromatography on Sephadex SPC-50 [10].

The peptide containing the cluster of phosphoserine residues was separated from the solution on tryptic digestion of β-casein by the barium precipitation method given by Manson and Annan [3]. It was further purified and the barium was removed by chromatography on Sephadex A25, using a gradient of pyridine/formate buffer, pH 2.7, increasing in concentration from 0.2 to 1.2 M. The fractions containing phosphopeptide were combined and the peptide was recovered by lyophilization and stored as a powder at −20° C. The product behaved as a single homogeneous peptide when subjected to electrophoresis on cellulose acetate in citric acid (250 mM) at pH 1.9 (200 V, 10 mA) and contained 3.4% phosphorus.

The bovine spleen phosphoprotein phosphatase enzyme was extracted and purified by modification of the procedure of Revel and Racker [11].

A fresh spleen was homogenised with four times its volume of 200 mM sodium acetate, pH 5.0, containing 300 mM NaCl. After centrifugation (2000 × *g*, 20 min), the supernatant was heated at 70° C for 3 min in batches and again centrifuged (2000 × *g*, 20 min). Following treatment with protamine sulphate (0.2% final concentration) and centrifugation (2000 × *g*, 20 min) to remove denatured protein, the enzyme was precipitated from the supernatant by addition of $(NH_4)_2SO_4$ to 80% saturation. It was purified by chromatography on car-

boxymethyl-Sephadex (CM50) at pH 5.0 and then rechromatographed on the same support at pH 6.0. The active fractions from the second column were concentrated by ultrafiltration and the enzyme was further purified by gel filtration chromatography on a column of Sephadex G-150. After concentration, the enzyme solution was stored at −20° C.

Dephosphorylation of the phosphopeptide was performed at room temperature in sodium acetate buffer, pH 5.8, at an enzyme to substrate ratio of 1 : 100 (w/w). Aliquots removed from the raction mixture at suitable time intervals were added to $HClO_4$ stop the reaction and the resultant solution were subjected to electrophoresis. Cellulose acetate electrophoresis of the peptide solutions were performed in a Shandon Universal Electrophoresis tank (Shandon Southern Instrument Limited, Camberley, Surrey). Cellulose acetate gels 7.8 × 15 cm (Cellogel, Reve Angel Scientific Ltd., Maidstone, Kent) were soaked in citric acid (250 mM, pH 1.9) and the same solution was used in the electrophoresis tank. 5-µl aliquots of the peptide solutions were applied to the gels using a microlitre syringe and the strips were run by applying a potential gradient of 50 V/cm for 20 min. The fully phosphorylated peptide was least mobile of the peptides but sequential removal of the phosphate groups progessively increased the mobility of the molecule. Completely dephosphorylated species travelled for 4 cm. This system provided excellent resolution of the mixture of partially dephosphorylated species and the bands were well separated and distinct. After electrophoresis the bands were visualised by staining for 5 min with Procion Blue H5R (B.D.H. Limited, Poole, Dorset) (0.1% (w/v) in methanol containing 2% (v/v) HCl). Background stain was removed by two washes (twice 5 min) in methanol. While still damp, the stained bands of peptide were cut from the gels and the dye-peptide complexes were eluted with 1 ml of alkaline methanol. The absorbances of the resulting solutions were measured at 570 nm in a Gilford model 240 spectrophotometer. Blank sections of cellulose acetate were also cut from between the peptide bands and treated in the same manner to allow for residual background stain.

Results and Discussion

When spleen phosphoprotein phosphatase was allowed to react with the phosphopeptide derived from β-casein, inorganic phosphate was liberated at a rapid rate until approx. 25% of the phosphate initially present had been released (Fig. 1). Subsequently, phosphopeptide continued to be removed but at a steadily declining rate. The relative proportion of the partially dephosphorylated peptides in aliquots of the reaction mixture taken at different times were determined and are presented in Fig. 2.

The first aliquots taken from the reaction mixture after 1 and 2 min show only the original phosphopeptide (P_4) * and that peptide derived from it by the loss of one phosphate group (P_3). P_2 first appeared 4 min after the start of the reaction although it was only 0.6% of the total peptide mixture at this stage. Both this peptide and the accompanying P_3 continued to increase in amount as the reaction proceeded simultaneously with the disappearance of the starting phosphopeptide. The proportion of P_3 was at a maximum in the 15-min aliquot whilst P_2 showed a similar but somewhat higher maximum 25 min later. A

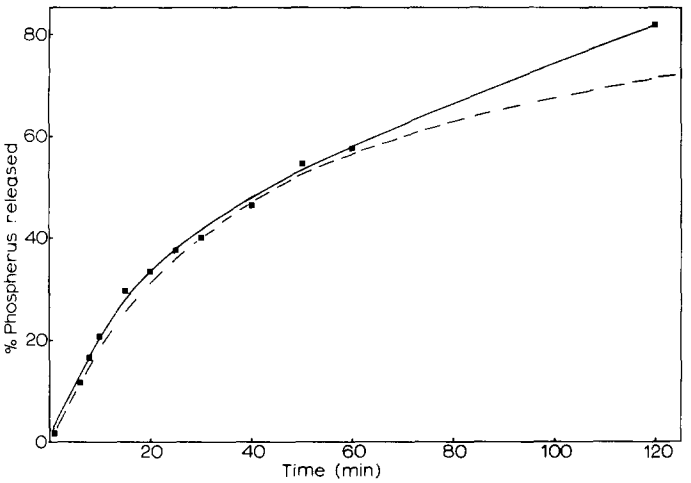

Fig. 1. The liberation of inorganic phosphate during the dephosphorylation of the phosphopeptide of β-casein. 15.4 mg of peptide, 91 μg of enzyme in 530 μl of sodium acetate buffer, pH 5.8, with mercaptoethanol (10 μmol). 30 μl aliquots were removed at appropriate times, the reaction was stopped and phosphate and peptide determinations were made. The dotted line is the calculated phosphate release using the sequential reaction scheme.

fourth band corresponding to P_1 was detectable on the electrophoretograms of the aliquot taken 20 min after the start of the reaction, although at this point the reaction mixture still contained 16% of the original phosphopeptide. However, all the molecules of the starting peptide had lost at least one phosphate group before completely dephosphorylated peptide was detectable in the reaction mixture. The reaction thus proceeded in an essentially sequential manner

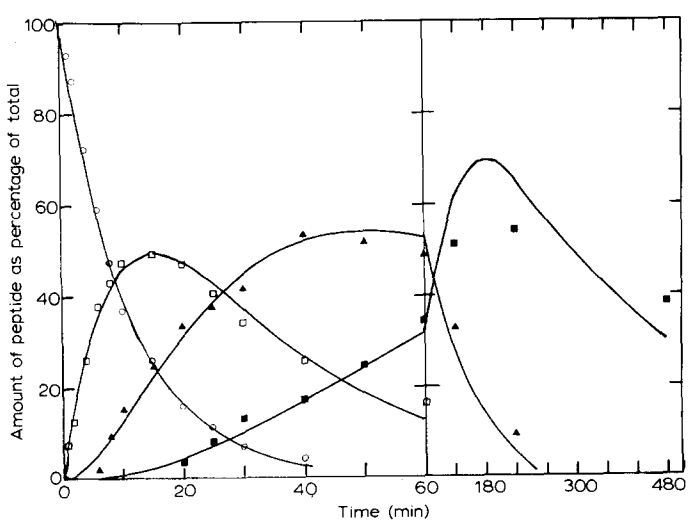

Fig. 2. Variation with time of the partially phosphorylated peptides formed during dephosphorylation of P_4. The amounts are expressed in terms of percentages of total peptide present. P_4, P_3, P_2 and P_1 are represented by ○, □, ▲ and ■, respectively. Solid lines show calculated amounts using the rate constants quoted in Table I.

so that each partially phosphorylated species rose to a maximum in turn and none of the species were particularly resistant to enzymic attack. Also, since completely dephosphorylated peptides did not occur in reaction mixtures that still contained fully phosphorylated material the enzyme did not act by removing all of the phosphates from one peptide molecule before passing to a second substrate molecule.

A plot of the disappearance of P_4 showed that its reaction was approximately first order (Fig. 3). Rate constants for the dephosphorylation of the other peptides were assumed to be pseudo first order also, and the sequence of reactions was considered to be

$$P_4 \overset{k_1}{\rightarrow} P_3 \overset{k_2}{\rightarrow} P_2 \overset{k_3}{\rightarrow} P_1 \overset{k_4}{\rightarrow} P_0$$

where P_4, P_3, P_2, P_1 and P_0 represent peptides containing 4, 3, 2, 1 and 0 phosphates, respectively. At any time the rate of decay of peptide P_x is given by the expression

$$\frac{d(P_x)}{dt} = k_f(P_{x+1}) - (k_d(P_x)$$

where k_f if the rate constant for the formation of P_x from P_{x+1} and k_d is the rate of decay of P_x. Substitution of the appropriate constants k_1, k_2, k_3 etc. provides a series of equations which can be sequentially integrated to give the concentrations of the various P_x as functions of time. The rate constants (Table I) were calculated originally by numerical solution of the equations, and were checked by finding the best least-squares fit of the integrated P_x vs. time relations to the experimental data, applying weighting to fit the earlier parts of the data, i.e. that obtained before 120 min. Plots of the calculated quantities P_x against time were compared with experimentally derived graphs (Fig. 2) from which it can be seen that whilst agreement was obtained for the early stages of

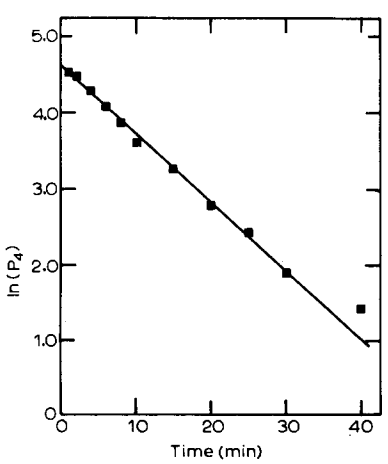

Fig. 3. First-order reaction of phosphopeptide P_4. Reaction conditions as for Fig. 1. This shows linear drop in ln [P_4] over 95% of the reaction.

TABLE I

RATE CONSTANTS FOR DEPHOSPHORYLATION OF THE PHOSPHOPEPTIDE OF β-CASEIN

Units are min^{-1}. A and B are the constants for specific and non-specific hydrolysis (see text).

	A	B
k_1	0.0900	0.0225
k_2	0.0458	0.0152
k_3	0.0154	0.0077
k_4	0.0027	0.0027

the reaction (k_1 and k_2) the computed curve deviates from the experimental results for the breakdown of P_2 and P_1; this is also apparent from the calculated rate of formation of inorganic phosphate (Fig. 1). This deviation could be due to two possibilities: (A) inaccurate estimation of the quantities of the various peptides present. Although the linearity of the plot of colour intensity against peptide concentration was checked for P_4, it was not possible to implement the same check with the partially dephosphorylated peptides since they were not available in sufficient quantity. Although Procion Blue forms a covalent linkage with proteins it is also known to participate in ionic bonding to a small extent and it is possible that removal of phosphates from the peptide could influence the amount of dye bound. (B) The simple sequential first-order reaction scheme is not adequate to describe the reaction (for instance inhibition by any of the products was not considered). The V and K_m for each of the substrates interacting with the enzyme will be required to describe the reaction fully and the problem of separating the intermediate peptides for this purpose is currently being investigated.

If the hydrolysis is a non-specific process, then the rate constants quoted must be divided by a statistical factor to cope with the number of phosphate groups per peptide, i.e. $k_1' = k_1/4$, $k_2' = k_2/3$ etc (Table I). If, however, the phosphates are removed in a definite sequence so that the hydrolysis may be considered as specific, then the treated rate constants quoted reflect the difference in the rates of hydrolysis of individual phosphates. Furthermore, the computed results do not take into account a number of other factors, particularly the binding constants of each reacting species with the enzyme.

The results obtained do not indicate as great a variation between the different rate constants as might be contemplated from the comparatively easy transfer of phosphate to ADP observed by Rabinowitz and Lipman [6]. The present investigation has, however, only been concerned with the transfer of phosphate to water and it is probably that in the cell other substrates are better acceptors than water. Nevertheless the preliminary results presented do affirm that interaction in the intact phosphopeptide results in an enhancement of the activity of the phosphoseryl cluster, thus providing additional support for the phosphate transfer reaction observed with β-casein.

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