PHOSPHORYLATION OF CASEIN FRACTIONS BY RAT LIVER 'PHOSVITIN KINASE'

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1. Introduction

Previous work from our laboratory [1] had shown that the 'phosvitin kinase' activity of crude rat liver cytosol is resolved by Sepharose 6B gel-filtration into two enzyme fractions. Both fractions, further purified by P-cellulose chromatography, still phosphorylate endogenous substrates, such as mitochondrial membrane proteins [2] as well as exogenous proteins like phosvitin and casein, but display only neglegible activity, if any, towards histones and protamines. However the two enzyme fractions, which are cAMP-independent, exhibit different catalytic activities since the less retarded and predominant Sepharose fraction phosphorylates both threonine (thr) and serine (ser) residues of whole casein, 'TS-form', while the other fraction is active only on serine residues, 'S-form' [1].

It should be noted that whole casein, used in the investigations on protein kinases, is a heterogeneous mixture of several different 'caseins'. The aim of this work is to study the phosphorylation of the single casein fractions and determine in which fractions are located the serine and threonine residues involved in the kinase reaction. The results of such an investigation, combined with the knowledge of the caseins' primary structure, might provide new information about the specificity of 'phosvitin casein–kinases' and the structural features that determine the phosphorylation of the proteins by these enzyme fractions.

2. Materials and methods

2.1. *Caseins*

'Whole casein' was Hammarsten Casein from Merck. α_1-Casein was a commercial product from Merck purified by DEAE-cellulose column chromatography, according to Ribadeau-Dumas et al. [3]. For some experiments however it was further purified by 7.5% polyacrylamide gel electrophoresis, pH 8.9, using the system described by Maurer [4], in which 5 M urea was included. The running time was twice that required for the marker dye (bromophenol blue) to reach the end of the column. Each gel column was then sliced into two longitudinal sections, one of which was stained for proteins with Coomassie Brilliant Blue [5]. The main fast moving band of α-casein, which was well separated from contaminating traces of κ- and β-caseins, was eluted from the corresponding segment of the unstained half of the gel by mincing it in distilled water.

κ-Casein was prepared following the Zittle and Custer procedure up to the ethanol precipitation step [6]. Its P-content, determined after digestion in perchloric acid, according to Wagner [7], was 0.26%. Its purity was tested by the same gel electrophoretic procedure just described for α-casein. Only the diffused band near the origin, typical for κ-casein [6], was evident with trace amounts of α- and β-caseins.

2.2. *'TS'- and 'S'-forms of cytosol phosvitin casein–kinase*

The procedure followed for the preparation of the two fractions of phosvitin casein–kinase, distinguishable by their different activity towards serine and threonine residues of casein, was very similar to that outlined in a previous paper [1], combining Sepharose 6B and P-cellulose chromatography. For a typical preparation the livers of 4 Wistar albino rats were homogenized in 100 ml of 0.25 M sucrose and

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centrifuged at 105 000 × *g* for 60 min. The clear supernatant was concentrated to about 8 ml by ultrafiltration through DiaFlo UM10 membranes, dialyzed overnight against 0.1 M Tris–HCl, pH 7.5, containing 0.5 M NaCl, and filtered through a Sepharose 6B column (1.9 × 120 cm) equilibrated with the same buffer and eluted with a flow rate of 5 ml/h. Under these conditions phosvitin kinase activity, tested as previously described [8], was resolved into two sharp and well separated peaks of which the less retarded peak is predominant (see also ref. [1]). These two fractions, after concentration by ultrafiltration and dialysis were separately adsorbed to a P-cellulose column (1.9 × 8 cm) equilibrated with 0.05 M Tris–HCl, pH 7.5, containing 0.25 M NaCl. Most of contaminating proteins were eliminated by washing the column with 200 ml of the equilibrium buffer, followed by 200 ml of the same buffer containing 0.3 M NaCl. Finally all phosvitin kinase activity was eluted from the column by raising the NaCl concentration to 0.70 M. The preparation from the less retarded Sepharose peak was consistently found to phosphorylate whole casein to give a [^{32}P]thr/[^{32}P]ser ratio ranging between 1.5 and 2.0 and is referred to as the 'TS-form'. The preparation from the more retarded and minor Sepharose peak, on the contrary, phosphorylates whole casein exclusively at its serine sites and is referred to as the 'S-form'.

2.3. *Enzymatic phosphorylation of casein*

For determining the rate of phosphorylation by phosvitin casein–kinase, 5 mg of whole casein or casein subfractions were incubated at 37°C for 10 min in 0.5 ml of a medium containing 100 mM Tris–HCl buffer, pH 7.5, 12 mM $MgCl_2$, 50 μM ATP, containing 0.5 μCi [γ-^{32}P]ATP (from Radiochemical Centre, Amersham) and 1–5 μg of either the TS- or the S-forms of the enzyme. The reaction was stopped by addition of 5 ml 10% trichloroacetic acid. The precipitated proteins were washed three times more by suspension in 5 ml of 10% trichloroacetic acid, followed by centrifugation and finally dissolved in 'Instagel' scintillation liquid and counted in a Packard liquid scintillator.

2.4. *Fractionation of ^{32}P-labelled whole casein*

Fractionation of ^{32}P-labelled whole casein was performed by submitting 40 mg of Hammarsten casein to DEAE-cellulose column chromatography, essentially according to Ribadeau-Dumas et al. [3]. After it has been previously labelled by 30 min incubation with [γ-^{32}P]ATP and the TS-form of phosvitin casein–kinase following the procedure described above, it was dissolved in 4 ml of 0.02 M Imidazole buffer, pH 7.0, containing 4.5 M urea and applied to a DEAE-cellulose column (1.9 × 8.5 cm). The column was equilibrated with 0.02 M Imidazole buffer, pH 7.0, containing 3.3 M urea and was eluted with a flow rate of 43 ml/h. The volume of the linear gradient, ranging from 0–0.4 M NaCl was 600 ml. Single fractions (3.3 ml) were analyzed for both their protein (A_{280}) and ^{32}P-content (by liquid scintillation of 0.2 ml aliquots).

2.5. *Identification of ^{32}P-labelled phosphoaminoacids*

Identification of ^{32}P-labelled phosphoaminoacids, was performed on casein fractions (1–5 mg) after partial acid hydrolysis (10 h in 2 N HCl at 105°C) by pH 1.9 paper electrophoresis, as previously described [2].

3. Results

Whole casein, previously labelled by phosphorylation with [γ-^{32}P]ATP in the presence of the TS-form of phosvitin casein–kinase, was resolved into its components by DEAE-cellulose chromatography with a linear concentration gradient of buffered NaCl, containing 3.3 M urea. The result of a typical experiment (fig.1), clearly shows that the optical density and radioactivity profiles are not overlapping, indicating the preferential phosphorylation of some protein fractions. In fact, the radioactivity associated with the absorbance peaks of γ- and β-caseins is negligible, while the two major radioactive peaks are eluted in the κ-casein region, i.e., between β- and α-casein [3] and overlapping the absorbance peak of α-casein. However the relative amounts of protein-bound [^{32}P]ser and [^{32}P]thr residues in these two radioactive peaks are quite different. As shown in fig.2, only [^{32}P]thr could be isolated from the acid hydrolysate of peak I (profile A), while [^{32}P]ser residues predominate in peak II (profile B), suggesting that threo-

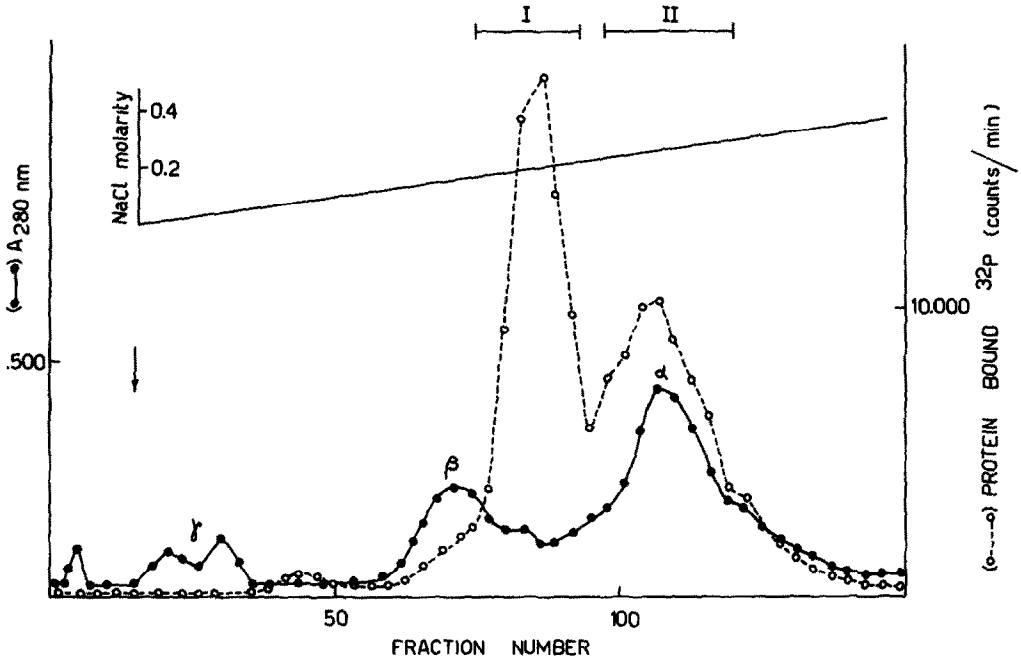
I
II
NaCl molarity
0.4
0.2
(●—●) A280 nm
.500
10.000
(o---o) PROTEIN BOUND 32P (counts/min)
α
β
γ
50
100
FRACTION NUMBER

Fig. 1

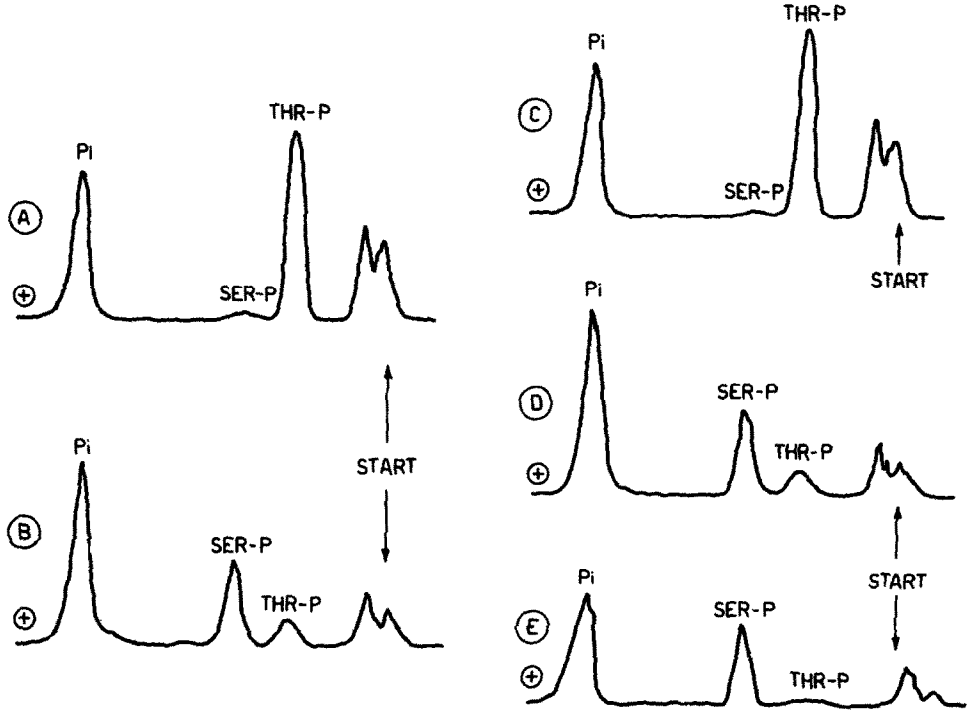
A
Pi
THR-P
SER-P
START
B
Pi
SER-P
THR-P
C
Pi
THR-P
SER-P
START
D
Pi
SER-P
THR-P
START
E
Pi
SER-P
THR-P

Fig. 2

nine residues are phosphorylated in κ-casein(*) and serine residues in α-casein. In order to get more definite evidence on this point κ-casein, prepared according to Zittle and Custer [6] and commercial α_1-casein purified by DEAE-cellulose chromatography according to Ribadeau-Dumas et al. [3] were separately phosphorylated by the TS-form of phosvitin casein–kinase and analyzed for their [^{32}P]phosphoaminoacids content. As shown in fig.2, the patterns obtained from labelled κ- (profile C) and α-casein (profile D) are quite different from each other and very similar to those from peaks I and II, respectively. This provides additional evidence that threonine residues are phosphorylated in κ-casein by the TS-form of phosvitin casein–kinase. The amount of this labelled phosphoaminoacid, in comparison with [^{32}P]ser, is almost negligible in the α-casein. Moreover, when the α-casein is further purified by polyacrylamide gel electrophoresis, [^{32}P]thr disappears from its electrophoretic pattern (fig.2, profile E), thus indicating that its occurrence in profile D was due to contaminating proteins.

It was interesting at this point to test the phosphorylation of κ- and α-casein by the S-form of phosvitin casein–kinase which, unlike the TS-form used in the above experiments, was shown not to phosphorylate threonine residues of whole casein [1]. Surprisingly, as shown in table 1, κ-casein is phosphorylated by the S-form, even more actively than α-casein.

*The possibility that radioactive peak I (fig.1) might consist of β-casein molecules phosphorylated de novo at threonine residue(s) and thereafter more retarded by DEAE-cellulose, is ruled out by the finding that the β-casein peak further purified by rechromatography on DEAE-cellulose column undergoes only negligible phosphorylation by the TS-form of phosvitin casein–kinase.

Table 1
Phosphorylation rates of κ- and α-caseins by phosvitin kinase (TS- and S-forms)

Enzyme-form	^{32}P-Incorporation (cpm)	
	κ-Casein	α_1-Casein
TS	57 800	5870
S	14 400	4900

Experimental conditions are described in the Materials and methods section

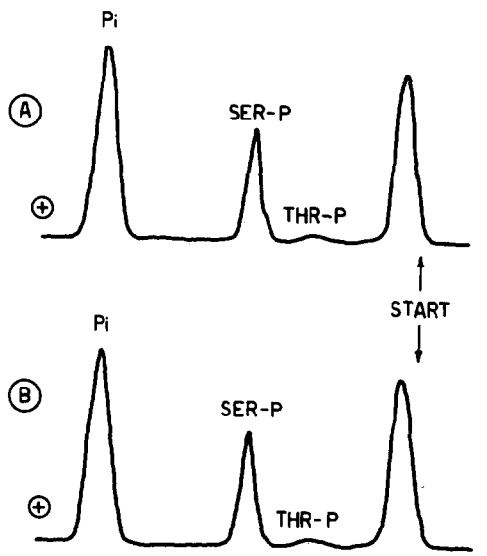

Fig.3. Isolation of [^{32}P]ser and [^{32}P]thr from κ- and α-caseins phosphorylated by the S-form of phosvitin kinase. General conditions for phosphorylation, hydrolysis and paper electrophoresis are described in the Materials and methods section. Profiles A and B refer to ^{32}P-labelled κ- and α_1-caseins, respectively.

Fig.1. Fractionation of ^{32}P-labelled whole casein by DEAE-cellulose chromatography. General conditions for the labelling of casein by the TS-form of phosvitin kinase and its fractionation are described in the Materials and methods section. At the arrow, the linear gradient of NaCl (0–0.40 M in 0.02 M Imidazole buffer, pH 7.0, containing 3.3 M urea) was started. The three absorbance peaks eluted by increasing ionic strength correspond to γ-, β- and α-caseins respectively. κ-Casein, eluted between β- and α-caseins [3] is not clearly indicated as an independent absorbance peak.

Fig.2. Isolation of [^{32}P]ser and [^{32}P]thr from different casein fractions phosphorylated by the TS-form of phosvitin kinase. General conditions for phosphorylation, hydrolysis and paper electrophoresis are described in the Materials and methods section. Electrophoretic profiles refer to: (A) radioactive peak I from DEAE-cellulose chromatography of whole [^{32}P]casein (collected as indicated in fig.1). (B) Radioactive peak II from DEAE-cellulose chromatography of whole [^{32}P]casein (collected as indicated in fig.1). (C) ^{32}P-labelled κ-casein. (D) ^{32}P-labelled α_1-casein. (E) ^{32}P-labelled α_1-casein after further purification by polyacrylamide gel electrophoresis (see Materials and methods).

However under these conditions only serine residues are phosphorylated in κ-casein (fig.3).

4. Discussion

The present paper concerns the phosphorylation of casein fractions by two forms of rat liver 'phosvitin casein–kinase', a protein kinase (EC 2.7.1.3.7.) apparently insensitive to cAMP, active on phosvitin, casein and endogenous substrates but not significantly active on protamines and histones. Two such enzyme forms had been previously characterized for their different behaviour towards whole casein: the 'TS-form' being active on both threonine and serine residues, while the 'S-form' phosphorylates only serine residues [1]. The preferential phosphorylation of serine and threonine residues of whole casein by different forms of protein kinases has been reported also by other authors [9]. However the heterogeneity of whole casein raised several questions about the susceptibility to enzymatic phosphorylation of the different casein fractions and the distribution of [^{32}P]ser and [^{32}P]thr among them.

The results reported in the present paper can be briefly summarized as follows:

(i) α-Casein is phosphorylated by both forms of the enzyme at serine residues

(ii) A casein fraction identified as κ-casein is phosphorylated by the TS-form at threonine residue(s) and by the S-form at serine residue(s)

(iii) The phosphorylation of the γ- and β-casein fractions in whole casein appears to be negligible compared with that of κ- and α-caseins.

Hence the previously reported phosphorylation of both serine and threonine residues of whole casein [1,9,10] by rat liver cytosol phosvitin kinase must be looked at as the phosphorylation of distinct substrates, involving the threonine residues of κ-casein and the serine residues of α-casein.

On the other hand the finding that κ-casein undergoes phosphorylation at different sites, namely threonine and serine residues, depending on the enzyme form used, suggests that the two forms of rat liver cytosol phosvitin kinase display a 'site specificity' rather than, or in addition to, a 'substrate specificity', since they recognize different sites in the same protein.

Finally its higher phosphorylation rate might also suggest that κ-casein represents a better model than other caseins for the phosphorylation process of mitochondrial membrane proteins which also are extensively phosphorylated by liver phosvitin kinase at threonine residues [2]. It is reasonable to speculate that structural similarities might indeed exist between mitochondrial membrane proteins and κ-casein which is a typical amphipathic protein, with a large hydrophobic moiety which can be expected to be easily embedded in a lipid structure like a membrane, and a short, relatively hydrophilic tail (the 'glicomacropeptide' released by rennin) [11] which can be expected to project into the aqueous medium and be phosphorylated by phosvitin kinase. It should also be recalled that most of the TS-form of phosvitin kinase is present outside the mitochondrial structures, in the soluble cytosol [1], where it would be able to phosphorylate only hydrophylic regions of the membrane proteins exposed to the surrounding soluble phase.

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