CASEIN PHOSPHATASE DEPENDENT DEPHOSPHORYLATION OF ^{32}P-CASEIN FRACTIONS LABELLED BY TWO PROTEIN KINASES

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Received July 8,1977

SUMMARY: The dephosphorylation by a rat liver "Casein phosphatase" of ^{32}P-casein preparations labelled by two protein kinases displaying different specificities for casein residues and subfractions has been studied. Although the Pi released by the phosphatase accounts for no more than 20% of the total casein bound P, it includes pratically all the ^{32}P incorporated into thr-^{32}P and ser-^{32}P residues by casein kinases TS and S respectively. Such radioactive residues preferentially hydrolyzed by the phosphatase are located in two different minor casein fractions distinct from the main α_{s1}, β and k-caseins. On the contrary the ser-^{32}P residues labelled by casein kinase TS and largely accounted for by α_{s1}-casein, are unaffected by the phosphatase. These results suggest that the minor casein fractions preferentially dephosphorylated by casein phosphatase share structural similarities with the still unknown endogenous substrate(s) of this enzyme.

INTRODUCTION

The substrate specificity of rat liver cytosol protein phosphatase (EC. 3.1.3.16) which is active on casein, but not on phosvitin or on phosphohistones, has been previously investigated (1,2). In particular, we have shown that such a soluble enzyme, unlike mitochondrial protein phosphatase, catalyzes a preferential hydrolysis of the thr-^{32}P residues of whole casein labelled by [γ^{32}P] ATP in the presence of a rat liver cytosol preparation of protein kinase (EC.2.7.1.37) which is able to phosphorylate both serine and threonine residues of whole casein (2). However the rat liver cytosol protein kinase preparation contains two forms of enzyme: a largely predominant casein kinase TS, phosphorylating both threonine and serine residues of casein; and a casein kinase S, active only on serine residues (3). Also, we recently demonstrated that the ser-^{32}P and thr-^{32}P residues of whole casein are located in different casein fractions (4,5). These findings prompted us to study the activity of cytosol casein phosphatase on the different casein fractions labelled by either casein kinase TS or S, in order to establish whether the previously observed preferential dephosphorylation of the thr-^{32}P residues is due to either an enzyme specificity for such a type of residues or, rather, for substrates fulfilling a critical structural requirement around the phosphorylated residues involved in the phosphatase reaction, independent of their chemical nature.

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ISSN 0006-291X

The experiments described in the present paper provide clear-cut evidence that the specificity of rat liver cytosol casein phosphatase is not determined by the nature of the phosphorylated residue per sè since ser-^{32}P residues phosphorylated by casein kinase S are dephosphorylated as actively as the thr-^{32}P residues phosphorylated by the casein kinase TS, though the ser-^{32}P residues labelled by this latter kinase are resistant to the phosphatase. Our present knowledge about the distribution of such three pools of casein bound ^{32}P within casein subfractions is consistent with the hypothesis that cytosol casein phosphatase displays a remarkable catalytic activity toward some minor casein fractions which are also preferentially phosphorylated by the two cytosol casein kinases. It is, therefore, conceivable to assume that there are structural similarities between such minor casein fractions and the endogenous substrates of cytosol casein phosphatase and kinases as yet still unknown.

METHODS AND MATERIALS

Enzymes - Rat liver casein phosphatase was prepared by DEAE-cellulose column chromatography of crude rat liver cytosol as previously described (2): the second peak of activity eluted from the column was employed for the present study.

Casein kinases TS and S were separated and purified from rat liver cytosol following a procedure previously described (4).

Radioactive substrates - ^{32}P labelled casein was prepared by incubating 80 mg of whole casein("Hammarsten casein" from Merck precipitated at pH 4.6 (5)) at 37° for 60 min. in the presence of either casein kinase TS (10 µg) or S (25 µg) in 2 ml of a medium also containing: 100 mM Tris-HCl buffer pH 7.5, 12 mM $MgCl_2$, 50 µM [γ-^{32}P]ATP (specific radioactivity 20 µCi/µmole). The reaction was stopped by addition of 0.5 ml of 50% trichloroacetic acid, and the precipitate protein was washed 4 times with 6 ml of 10% trichloroacetic acid. The ^{32}P labelled precipitated casein was then dissolved in about 2 ml of distilled water by raising the pH to neutrality by addition of NaOH and dialyzed twice against 2 liters of 5 mM Tris-HCl pH 7.2.

For the labelling of α-casein the same procedure was followed except that whole casein was replaced by α_1-casein from Merck, consisting of over 90% α_{s1}-casein, according yo its polyacrylamide gel electrophoretic pattern.

"Thr-^{32}P Rich Fraction" and "Ser-^{32}P Rich Fraction" were prepared and labelled by submitting to phosphorylation by either casein kinase TS or S, the fractions 3 and 4 respectively from DEAE cellulose chromatography of unlabelled whole casein according to a previous paper (5).

Casein phosphatase determinations - Casein phosphatase activity was determined as ^{32}Pi released from the radioactive substrate by the enzyme at pH 6.0 (2). Incubation times are indicated in the individual tables and figures.

In some experiments the enzyme activities toward phosphorylserine (ser-^{32}P) and phosphorylthreonine (thr-^{32}P) were separately evaluated by isolating these phosphoaminoacids after 4 hrs hydrolysis at 105° in 6 N HCl of ^{32}P-caseins, preincubated with the phosphatase, by pH 1.5 paper electrophoresis, as previously described (6). For quantitative determinations, the radioactivity of the paper segments containing the phosphoaminoacids was extracted with 8 ml of "Insta Gel" scintillation liquid and counted in a liquid scintillator.

Separation methods - DEAE-cellulose chromatography of ^{32}P-casein before

Table I - Casein phosphatase dependent hydrolysis of ser-^{32}P and thr-^{32}P residues of ^{32}P-caseins labelled by two different casein kinases.

Labelling casein kinase:	Total P hydrolyzed %	^{32}P (cpm) hydrolyzed %	Ser-^{32}P(cpm)in: casein	dephospho-casein	Thr-^{32}P(cpm)in: casein	dephospho-casein
TS	16	58	8,150	7,900	11,800	580
S	16	89	19,225	2,110	absent	absent

The preparation of ^{32}P-caseins and the determinations of casein bound radioactive phosphoaminoacids were performed as described in the Method section. Dephosphocaseins were obtained by 60 min incubation of ^{32}P-caseins with casein phosphatase; control caseins were incubated under identical conditions omitting the enzyme.

and after incubation with casein phosphatase was performed essentially according to Ribadeau Dumas et al. (7) using a 1.9x8.0 cm column equilibrated with 0.02 M imidazole-HCl buffer pH 7.0 containing 4.5 M urea and eluted with 500 ml of a NaCl gradient (0.00 to 0.40 M) in 20 mM imidazole pH 7.0 containing also 3.3 M urea. The flow rate was 45 ml/hr, and the fractions (3.4 ml) were analyzed for their protein (A_{280nm}) and ^{32}P content (by liquid scintillation of 0.2 ml aliquots).

7.5% polyacrylamide gel electrophoresis, pH 8.9, was performed essentially according to Maurer (8). 5 M urea was also included, and the runs lasted 2.5 hrs, i.e. about one hr. more than the time required for the marking dye (Bromophenol Blue) to reach the end of the columns. After visualizing the casein bands with "Stains all" (9), the gel columns were sliced into two longitudinal sections one of which was inserted in a glass through 5 mM wide and 3 mm deep and scanned for its radioactivity in a Packard Radiochromatogram Scanner mod. 720.

RESULTS

The data reported in Table I clearly indicate that cytosol casein phosphatase, while dephosphorylating only a minor aliquot of the total protein-bound phosphate of whole casein, is very effective toward the radioactive pools of ^{32}P incorporated into whole casein by both casein kinases TS and S. The extent of dephosphorylation approaches 60% and 90% respectively. It should be noted, however, that the amino acid residues involved in the two dephosphorylation processes are different; namely thr-^{32}P residues with kinase TS and ser-^{32}P with kinase S. In contrast, those ser-^{32}P residues which are labelled b the casein kinase TS appear to be unaffected by the phosphatase, thus confirming a previous report (2).

The above results are in good agreement with the experiment illustrated in Fig. 1A. Whole ^{32}P casein labelled by casein kinase TS is resolved by DEAE cellulose column chromatography into two main radioactive fractions. The peak, marked A_1, which eluted between the β- and α_s-casein and which accounted for most

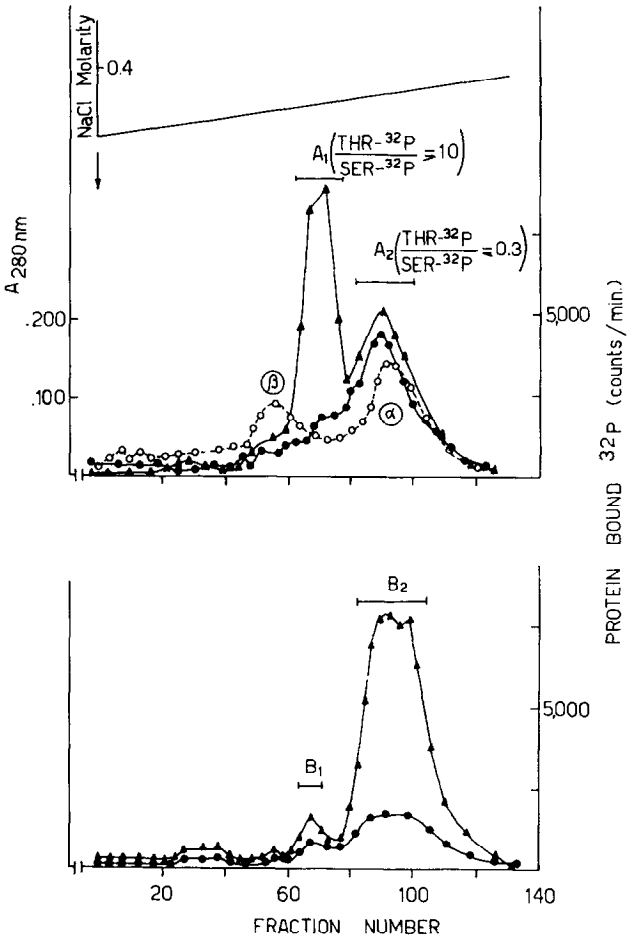

Fig. 1 - Effect of the preincubation with casein phosphatase on the radioactivity profiles obtained by submitting ^{32}P-casein (30 mg) labelled by either casein kinase TS (upper figure) or S (lower figure) to DEAE-cellulose column chromatography.

General conditions for the preparation of ^{32}P-caseins and for DEAE-cellulose fractionation are described in the Methods section. At the arrow the NaCl gradient was started. Profiles refer to: o- - -o A_{280nm} (omitted in the lower figure being superimposable to that of the upper figure); ▲——▲ ^{32}P-casein, control (preincubated in the medium for protein phosphatase without the enzyme); ●——● ^{32}P-casein preincubated 30 min with casein phosphatase. Both control and dephosphorylated ^{32}P-caseins were recovered at the end of incubation by 12% trichloroacetic acid precipitation, dissolved in the minimal volume of distilled water at neutral pH and dialyzed overnight against 2 liters of the equilibrium buffer of the column. Ser-^{32}P and thr-^{32}P were determined in the radioactive fractions as previously described (5).

if not all the thr-^{32}P residues incorporated, almost completely disappeared after incubation with the phosphatase. The more retarded radioactive peak (A_2), on the contrary, which roughly overlapped the α_S-casein main OD peak, and which

accounted mainly for the ser-^{32}P residues, is almost unaffected by incubation with casein phosphatase. However ser-^{32}P residues labelled in whole casein by casein kinase S, are promptly hydrolyzed by casein phosphatase (Fig.1B) and they are located in a casein fraction (B_2) displaying a DEAE-cellulose chromatographic behaviour similar to the ser-^{32}P rich fraction A_2 labelled by casein kinase TS, but this fraction is resistant to the phosphatase.

A priori this finding might be explained by assuming that the ser-^{32}P residues labelled by the two kinases are located in the same or in different casein subfractions which might well coexist in the heterogeneous "Ser-^{32}P Rich Fractions" (A_2 and B_2).Gel electrophoretic analysis of such ser-^{32}P rich fractions labelled by either casein kinase TS or S suggests that probably both phenomena contribute to explain their different sensitivities to casein phosphatase. The two radioactivity profiles, in fact, reported in Fig. 2, are partially, but not completely overlapping since the radioactivity incorporated in the presence of the kinase TS and resistant to the phosphatase is accounted for by both α_{s1}-casein and a broadened peak just following it, while only this latter broadened fraction, and not α_{s1}-casein, is radioactive when the casein kinase S was the labelling enzyme.

It should be concluded, therefore, that α_{s1}-casein, i.e. the main component of whole casein, is phosphorylated by the only casein kinase TS at serine residues which are unaffected by cytosol casein phosphatase, while some minor casein fractions, displaying the same gel electrophoretic behaviour as caseins of the α_{s2} group, are phosphorylated by both casein kinase TS and S, but, apparently, at different serine residues, since they are promptly dephosphorylated by the phosphatase only when the labelling enzyme was casein kinase S.

A confirmation of the insensitivity of α_{s1}-casein to cytosol casein phosphatase is provided in Table II where the dephosphorylation of isolated α_{s1}--casein previously labelled by casein kinase TS is compared with that of the isolated "Thr-^{32}P Rich Fraction" (TRF) and "Ser-^{32}P Rich Fraction" (SRF) labelled either by casein kinase TS or S. It can be seen that ^{32}P-α_{s1}-casein as well as the casein kinase-TS-labelled ser-^{32}P rich fraction are almost unaffected by the phosphatase, which, on the contrary, extensively dephosphorylates both the thr-^{32}P residues labelled by the casein kinase TS in the TRF and the ser-^{32}P residues of the SRF provided that the phosphorylating enzyme was casein kinase S.

DISCUSSION

The data reported in the present paper provide evidence of a marked specificity of rat liver cytosol casein phosphatase - a protein phosphatase inactive on both phosvitin and phosphohistones (2) - toward both thr-^{32}P and ser-^{32}P residues of ^{32}P-whole casein labelled by two protein kinases isolated from the sa-

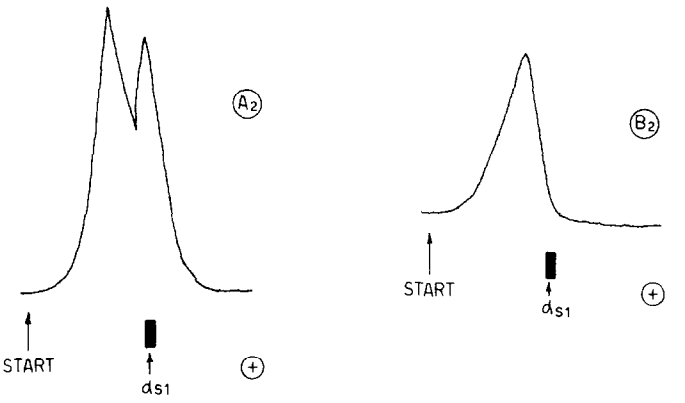

Fig. 2 - Polyacrylamide gel electrophoresis of the "Ser-^{32}P rich Fractions" A_2 and B_2 from DEAE-cellulose column of ^{32}P-casein labelled by casein kinase TS and S respectively.

Radioactive fractions A_2 (resistant to casein phosphatase) and B_2 (hydrolyzed by casein phosphatase) were obtained and collected as described in Fig. 1. Conditions for gel electrophoresis are described in the Method section.

me source, i.e. casein kinases TS and S (3). The phosphatase, though hydrolyzing no more than 20% of the total phosphate present in whole casein, splits virtually all the thr-^{32}P and ser-^{32}P residues labelled by casein kinase TS and S respectively. On the other hand, the ser-^{32}P residues labelled by the former kinase, and largely accounted for by α_{s1}-casein, represent the only radioactive casein-bound pool of phosphate insensitive to the phosphatase. The location of the thr-^{32}P and ser-^{32}P residues preferentially cleaved by casein phosphatase in a few minor casein fractions, distinct from the main casein components and here conventionally indicated as "Thr-^{32}P Rich Fraction" and "Ser-^{32}P Rich Fraction", advances the solution to the problem of their identification. In particular, it should be recalled that the previously suggested identification of the former fraction with k-casein (4) has been definitely ruled out by further chromatographic experiments (5). The behaviour of such a fraction on polyacrylamide gel electrophoresis at pH 8.9 is that of an homogeneous radioactive band running slightly ahead of β-casein (unpublished data), similar to the minor casein component "0.86" according to the nomenclature of Wake and Baldwin (10). On the other hand, the gel electrophoretic behaviour of the Ser-^{32}P Rich Fraction (see Fig. 2b) is typical for that group of minor α_s-caseins, more retarded than α_{s1}-casein (11) and referred to as α_{s2}(12).

It should be concluded, therefore, that rat liver cytosol casein phosphatase and, to a lesser extent, also casein kinases, display a remarkable specificity for some minor caseins which might hence be assumed to share structural

Table II - Casein phosphatase dependent dephosphorylation of casein subfractions labelled by two different casein kinases.

Casein Fraction:	Labelling casein kinase	^{32}P (cpm) hydrolyzed (% of total)
^{32}P- α_{s1}-casein	TS	3
"Thr-^{32}P Rich Fraction" (TRF)	TS	97
"Ser-^{32}P Rich Fraction" (SRF)	TS	12
" " " " "	S	85

The preparation of the radioactive fractions and their enzymatic dephosphorylation by 60 min incubation with casein phosphatase, were performed as described in the Methods section.

similarities with the still unknown endogenous substrates of these enzymes. From this point of view the elucidation of the primary structures of such caseins fractions, which is already in progress for α_{s2}-caseins (12), and their comparison with those of the main casein fractions - α_{s1},β and k-, which are already known (13-15), will be helpful for the understanding of the structural requirements and possibly of the biological role of these enzymes. The radioactivity incorporated by casein kinase TS into the Ser-^{32}P Rich Fraction, including α_{s1}-casein, is almost completely accounted for by polyphosphorylserine blocks (ser-^{32}P)n (5), thus suggesting that the phosphorylation of α_{s1}-casein involves its $(ser\text{-}P)_3$ sequence between ile_{65} and glu_{69}. The present finding that such a protein bound ^{32}P is the only one resistant to cytosol casein phosphatase, together with the previously reported failure of this enzyme to dephosphorylate phosvitin (2), a phosphoprotein outstandingly rich in $(ser\text{-}P)_n$ blocks (16), would be consistent with the assumption that such acidic blocks represent a structural hindrance to the action of cytosol casein phosphatase, but not against mitochondrial protein phosphatase, which is very active on phosvitin (17) and on casein ser-^{32}P residues labelled by casein kinase TS (2).

ACKNOWLEDGEMENTS

The Authors gratefully achnowledge Mrs. Giuliana Giungarelli for secretarial aid and Mr. Giorgio Parpajola for drawing of the graphs.

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