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DIFFERENT SUSCEPTIBILITY OF WHOLE CASEIN COMPONENTS TO ENZYMATIC PHOSPHORYLATION BY TWO FORMS OF RAT LIVER 'CASEIN KINASE'

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Summary

The phosphorylation of the single casein subfractions occurring when whole casein is incubated with [γ-^{32}P]ATP in the presence of two different rat liver 'casein kinases', both cyclic AMP-insensitive, has been studied.

"Casein kinase TS", active on both threonine and serine residues of whole casein, was found to be active towards a minor protein fraction, running slightly ahead of β-casein during gel electrophoresis, and accounting for most, if not all, of the [^{32}P]Thr residues labeled in whole casein ("[^{32}P]Thr-rich fraction"). The [^{32}P]Ser residues labeled by this enzyme were recovered in an heterogeneous "[^{32}P]Ser-rich fraction" including α_{s1}-casein together with minor α_s fractions, following α_{s1}-casein during gel electrophoresis.

"Casein kinase S", on the other hand, active only towards serine residues of whole casein, is active almost exclusively towards the minor α_s casein fractions, with the exclusion of both the "[^{32}P]Thr-rich fraction" and α_{s1}-casein itself.

Therefore, of the major casein components, β- and κ-caseins apparently play a quite unimportant role in the overall phosphorylation of whole casein by both the protein kinases tested, while α_{s1}-casein itself, unlabeled by casein kinase S, accounts for no more than 20—30% of ^{32}P incorporated in the presence of casein kinase TS.

Introduction

Although whole casein has often been used as a substrate for in vivo studies on protein kinases and phosphatases, very little is known about the involvement in the enzymatic reactions of the individual fractions of the heterogeneous preparation referred to as "acid" or "whole" casein. Recently we have purified from rat liver two protein kinase (EC 2.7.1.37) forms phosphorylating whole casein to give different [^{32}P]Ser/[^{32}P]Thr ratios [1] and we have then

provided evidences that [^{32}P]Ser and [^{32}P]Thr residues phosphorylated by one of these kinases — the so-called TS-forms — are located in different casein fractions [2].

In the present paper, the nature of the casein fractions involved in the phosphorylation process catalyzed by the two casein kinase forms is investigated.

Experimental Procedures

"Whole casein" was Hammarsten casein (Merck) which was dissolved at 10% in water (at neutral pH) and precipitated at pH 4.6 by 1 M HCl. The precipitate was dissolved in dilute NaOH and stored at −18°C.

α_1-casein was from Merck. By gel electrophoresis it gave a main band in the position of α_{s1} casein, accounting for more than 90% of the protein, and minor bands, made clearer by overloading the gel, in the positions expected for β-casein and between β and α_{s1}-casein.

"Casein kinases". The procedure followed for the separation and purification of the two rat liver cytosol casein kinases, referred to as "TS"- and "S"-forms has been previously described [2].

Preparation of ^{32}P-labeled caseins. For a typical experiment 80 mg whole casein were incubated at 37°C for 60 min in the presence of either the TS- (10 μg) or the S-form (25 μg) of casein kinase in 2 ml medium, also containing: 100 mM Tris · HCl buffer (pH 7.5), 12 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (specific activity 20 Ci/mol). The reaction was stopped by addition of 0.5 ml 50% trichloroacetic acid and the precipitated protein was washed 5 times with 6 ml 10% trichloroacetic acid. The ^{32}P-labeled precipitated casein was at this stage dissolved in about 2 ml distilled water at neutral pH by addition of NaOH solution.

Fractionation of ^{32}P-labeled whole casein. ^{32}P-labeled whole casein phosphorylated by either the TS- or S-forms of casein kinase was fractionated by DEAE-cellulose gradient chromatography in the presence of urea and mercaptoethanol, essentially following the procedure of Mercier et al. [3]. For a typical experiment 30 mg [^{32}P]casein were dialysed overnight against 1 l 0.02 M imidazole · HCl buffer (pH 7)/3.3 M urea/0.3% mercaptoethanol and applied to a DEAE-cellulose column (1.9 × 8.5 cm) equilibrated with the same buffer. After washing the column with 50 ml equilibrium buffer, a 500-ml gradient was started, ranging from 0 to 0.25 M NaCl in the same buffer. The column was finally eluted with the equilibrium buffer containing 0.25 M NaCl. The flow rate was approx. 50 ml/h. 3.6-ml fractions were collected which were analysed for both their protein content (A_{280nm}) and their radioactivity (by counting 0.3-ml aliquots in a liquid scintillator). The κ-casein elution position was determined by chromatography of a sample of κ-casein prepared according to the method of Zittle and Custer [16].

Gel chromatography. Sephadex G75 gel filtration of ^{32}P-labeled casein fractions from DEAE-cellulose, and of α_1-[^{32}P]casein was performed on a 1.8 × 79 cm column equilibrated with 15% formic acid and operated at a flow rate of 8 ml/h (3 ml fractions), which were analysed for their protein content (A_{280nm}) and radioactivity (by counting 0.2—0.5 ml aliquots in a liquid scintillator).

7.5% polyacrylamide gel electrophoresis. This was in 5 M urea at pH 8.9 as

previously described [2]. The runs lasted 2.5 h, i.e. about 50 min more than the time required for the marker dye Bromophenol Blue to reach the end of the column. Parallel standard runs were performed with α_{s1}-casein, β-casein (purified by repeated DEAE-cellulose chromatography [2]) and κ-casein prepared according to the method of Zittle and Custer [16]. Under our conditions, the main bands of β- and α_{s1}-caseins moved approx. 2.3 and 4.0 cm, respectively.

^{32}P-labeled phosphorylserine and phosphorylthreonine. These were isolated from whole casein and casein fractions after 4 h hydrolysis in 6 M HCl at 110°C by pH 1.9 paper electrophoresis, as previously described [4].

Proteins. The method was that of Lowry et al. [5] using a standard curve obtained with whole casein.

Results

(1) Fractionation of ^{32}P-casein phosphorylated by the two different casein kinases

The fractionation of whole casein previously labeled in the presence of [γ-^{32}P]ATP by either the TS- or the S-form of rat liver casein kinase was performed by DEAE-cellulose column chromatography in the presence of urea and mercaptoethanol. Such a procedure [3] was preferred to that [6] employed in a previous paper [2] since it allows a clear-cut separation of κ-casein from the α_s and β groups. The profiles obtained are reported in Fig. 1, A and B.

Fig. 1A, referring to whole casein labeled by the TS-form, indicates that most of the radioactivity was concentrated into two major peaks, preceding (A_1) and overlapping (A_2), the main absorbance peak of α_s-casein, respectively. The radioactivity associated with κ-, γ- and β-caseins was almost negligible.

The radioactivity pattern obtained with whole casein labeled by the S-form of the kinase (Fig. 1B) was characterized by the almost complete absence of the less-retarded radioactive peak (B_1) while the large radioactive peak overlapping the α_s main absorbance peak was still present. Again the radioactivity associated with κ-, γ- and β-caseins was almost negligible.

The radioactive fractions labeled by the two forms of casein kinase and resolved by DEAE-cellulose chromatography were collected as indicated in Fig. 1A and B and analysed for their [^{32}P]Ser to [^{32}P]Thr ratio in comparison with whole casein labeled by the same enzyme forms (Fig. 2). It can be seen that the radioactive fractions, A_1 and A_2, into which whole [^{32}P]casein labeled by the TS-form is resolved, displayed different relative amounts of [^{32}P]Ser and [^{32}P]Thr: namely, the radioactivity of the less retarded fractions (A_1) is almost completely accounted for by [^{32}P]Thr and thus referred to as the "[^{32}P]Thr-rich fraction" (TR fraction), while, correspondingly, the more retarded fraction (A_2), referred to as the "[^{32}P]Ser-rich fraction" (SR fraction), displayed a [^{32}P]Ser to [^{32}P]Thr ratio much higher than that of whole casein itself.

The patterns obtained with the DEAE-cellulose subfractions of casein labeled by the S-form are similar, in that the few [^{32}P]Thr residues labeled under these conditions and probably accounted for by slight contaminations of

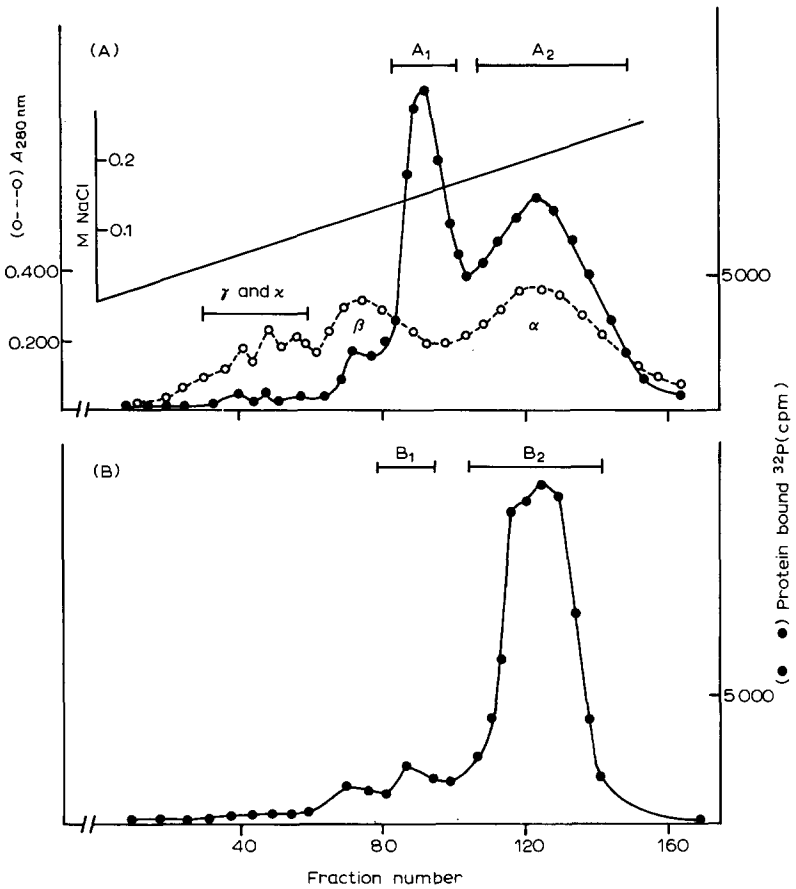

Fig. 1. DEAE-cellulose fractionation of ^{32}P-labeled whole casein labeled by the TS- (upper figure) and S-forms (lower figure) of casein kinase. The preparation of [^{32}P]caseins and the conditions for column chromatography are described under experimental procedures. The absorbance profile of the lower figure, about superimposable on that of the upper figure, has been omitted. Fractions were pooled as indicated, concentrated to about 5 ml by ultrafiltration and dialysed against 5 mM Tris · HCl pH 7 in order to remove urea.

the S-form by the TS-form, to be expected during the purification procedure [2], were found in the minor fraction B_1. This corresponds to fraction A_1 in Fig. 1A, being eluted just ahead of the α_s-casein main peak. The largely predominant and heterogeneous B_2 fraction was devoid of such residues (Fig. 2).

The above results indicate that most of the phosphorylation by the two casein kinase forms is accounted for by two casein subfractions: the TR fraction, significantly phosphorylated only by the TS-form, eluted between β and α_s-casein main absorbance peaks, and the SR fraction, roughly overlapping the α_s-casein main absorbance peak, evidently heterogeneous and phosphorylated by both the TS- and the S-forms, though not necessarily at the same sites or even on the same protein molecules.

(2) Characterization of the TRF

The DEAE-cellulose profile of Fig. 1 shows that the TR fraction (correspond-

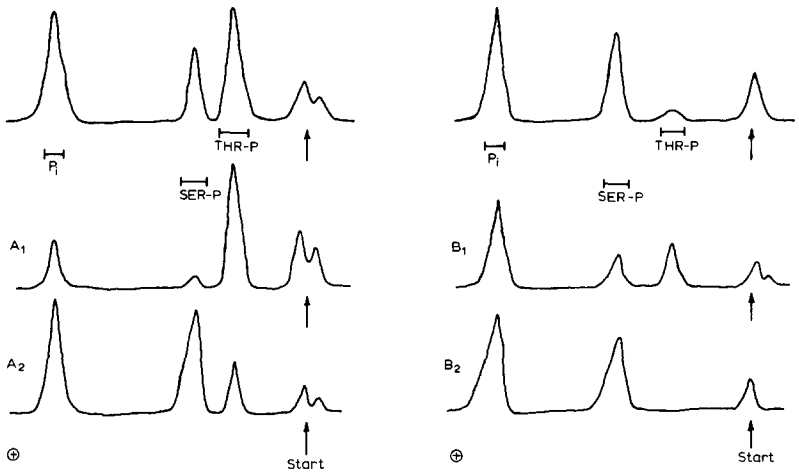

Fig. 2. Isolation of [^{32}P]Ser and [^{32}P]Thr from ^{32}P-labeled whole caseins and casein subfractions recovered from DEAE experiments as indicated in Fig. 1. The top profiles refer to whole [^{32}P]casein labeled by casein kinase TS (left) and S (right) respectively.

ing to peak A_1) of whole casein labeled by the TS-form, being eluted from the column between β- and α-casein fractions, was necessarily contaminated by them, mainly by β-casein. Thus, in order to get more information about its

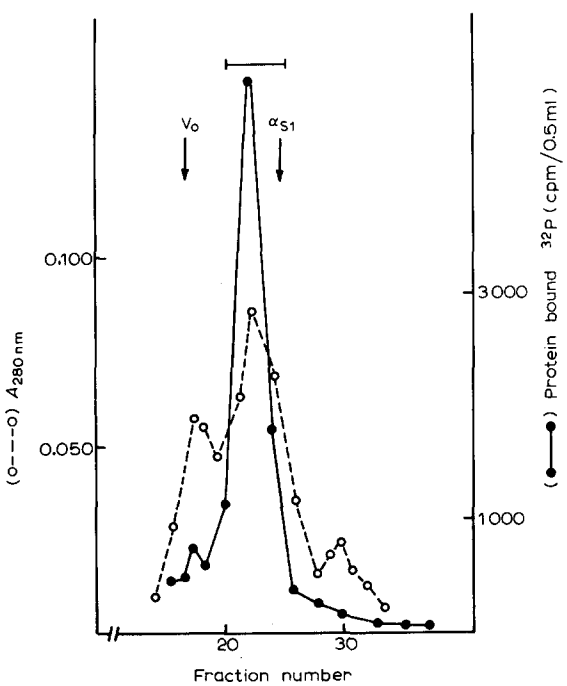

Fig. 3. Sephadex G75 chromatography of [^{32}P]Thr rich fraction (TR fraction). 5 mg of TRF A_1 (see Fig. 1) dissolved in 2 ml of 15% formic acid were submitted to gel filtration as described under Experimental Procedures.

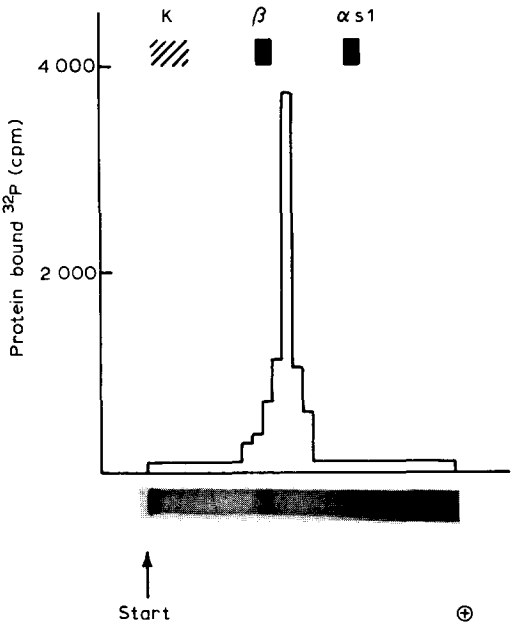

Fig. 4. 7.5% Polyacrylamide gel electrophoresis in 5 M urea pH 8.9 of 500 μg of [^{32}P]Thr rich fraction recovered from a Sephadex G75 column as indicated in Fig. 3, freeze-dried and dissolved in 0.1 ml of the electrophoresis buffer. After the run the gel column was stained for phosphoproteins with "Stains all" [8], photographed and sliced into 2-mm segments which were extracted for 4 h with 8 ml Instagel before counting in a liquid scintillator. The identification of the major protein band with β-casein was confirmed by parallel runs of whole casein.

nature, it was further purified by Sephadex G75 gel filtration followed by polyacrylamide gel electrophoresis.

Fig. 3 shows that gel filtration removed unlabeled or poorly labeled proteins (eluted at the V_0) from the major radioactive fraction, which was slightly retarded.

This latter fraction, however, was still contaminated by β-casein as shown on polyacrylamide gel electrophoresis (Fig. 4). It can be seen that the radioactivity was not homogeneously distributed throughout the large β-casein band, but was concentrated in a minor component running slightly ahead of it. The identification of such a TR fraction as an extraphosphorylated form of β-casein is ruled out by the finding [2], that it cannot be obtained from purified preparations of β-casein incubated with casein kinase.

(3) Characterization of the SR fraction

Unlike the TR fraction the SR fraction (i.e. A_2 and B_2 of Fig. 1, A and B, respectively) labeled in whole casein by both forms of casein kinase, is clearly heterogeneous. Once the SR fraction obtained by DEAE-cellulose chromatography of whole casein labeled by the TS- and S-forms was submitted to Sephadex G75 gel filtration, it gave rise to two patterns (Fig. 5), consisting of two well-separated peaks. One was eluted at the V_0, while the other was significantly retarded and exactly overlapped the main absorbance peak due to α_{s1}-

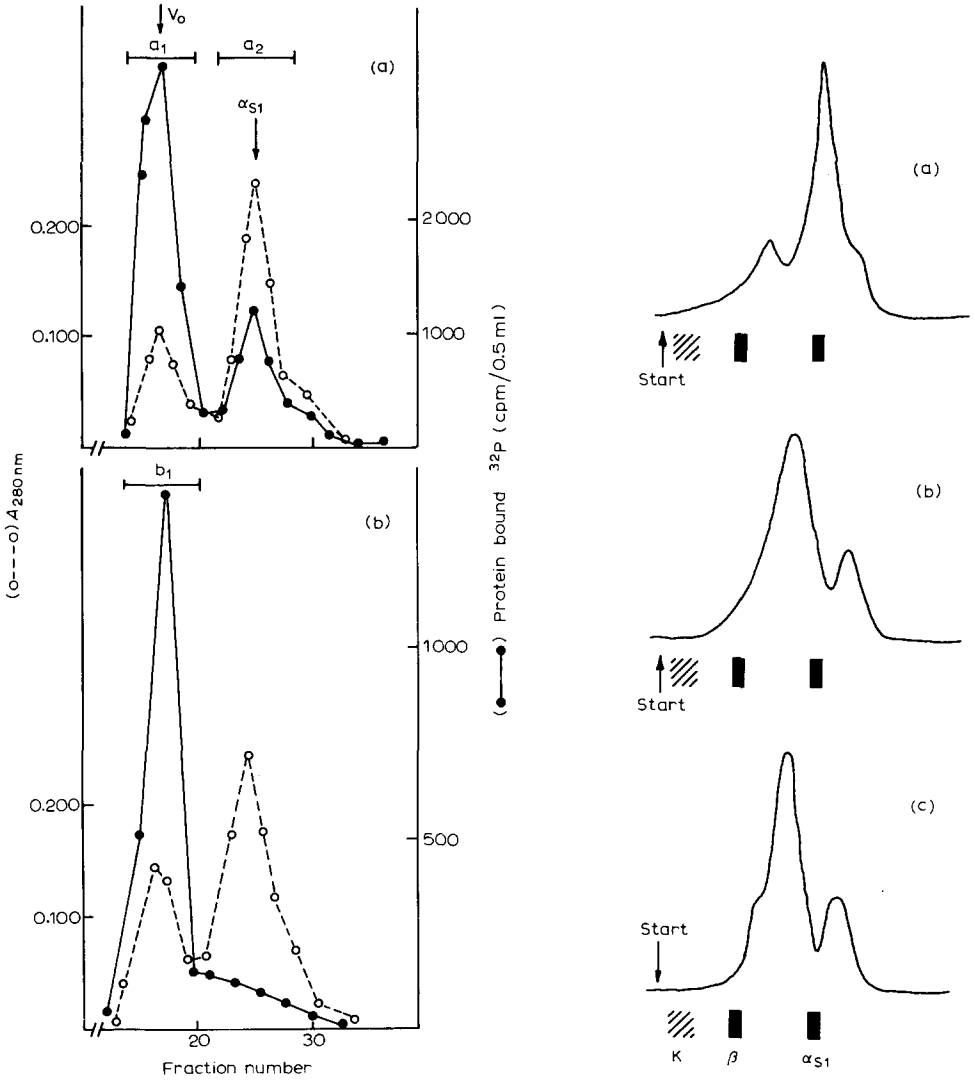

Fig. 5. Sephadex G75 chromatography of [^{32}P]Ser rich fractions (SR fraction) from ^{32}P casein phosphorylated by casein kinase TS (upper figure) and S (lower figure). 5 mg of the SRF A_2 and B_2 (see Fig. 1) dissolved in 2 ml of 15% formic acid were submitted to gel filtration as described in the experimental section.

Fig. 6. 7.5% Polyacrylamide gel electrophoresis in 5 M urea pH 8.9 of the [^{32}P]Ser rich subfractions (500 μg) resolved by Sephadex G75 chromatography. The single subfractions, collected as indicated in Fig. 5, were dissolved, after lyophilisation in the minimal volume of the electrophoresis buffer, and submitted to gel electrophoresis together with parallel samples of authentic α_{s1} casein. After the run each gel column was sliced into two longitudinal sections one of which was stained with Coomassie Brilliant Blue [9], to localize the α_{s1}-casein band while the other, in specially devised glass trough 5 mm wide and 3 mm deep, was scanned for its radioactivity in a Packard Radiochromatogram Scanner (Model 7201). Patterns a, b and c refer to peaks a_2, a_1 and b_1 of Fig. 5 respectively.

casein. The former showed the higher specific radioactivity and was phosphorylated by both the TS-form (Fig. 5a) and the S-form (Fig. 5b), while the latter, displaying the lower specific radioactivity when the phosphorylating

enzyme was the TS-form, was practically unlabeled when the phosphorylation was performed with the S-form.

This retarded fraction was identified with α_{s1}[^{32}P]casein by polyacrylamide gel electrophoresis (Fig. 6a). Its radioactivity was found to be accounted for by [^{32}P]Ser under the experimental conditions employed.

Fig. 6 also shows the polyacrylamide gel electrophoresis profiles of the fractions with higher specific radioactivity, eluted from Sephadex G75 at the V_0 deriving from the SR fraction of whole casein labeled by the TS-form (Fig. 6b) and the S-form (Fig. 6c) of casein kinase. It can be seen that the two profiles are substantially similar, both consisting of a main broad, irregular radioactive peak, more retarded than α_{s1}-casein and sometimes accompanied by a minor peak running ahead of α_{s1}-casein. The sharpness and overall mobility of the

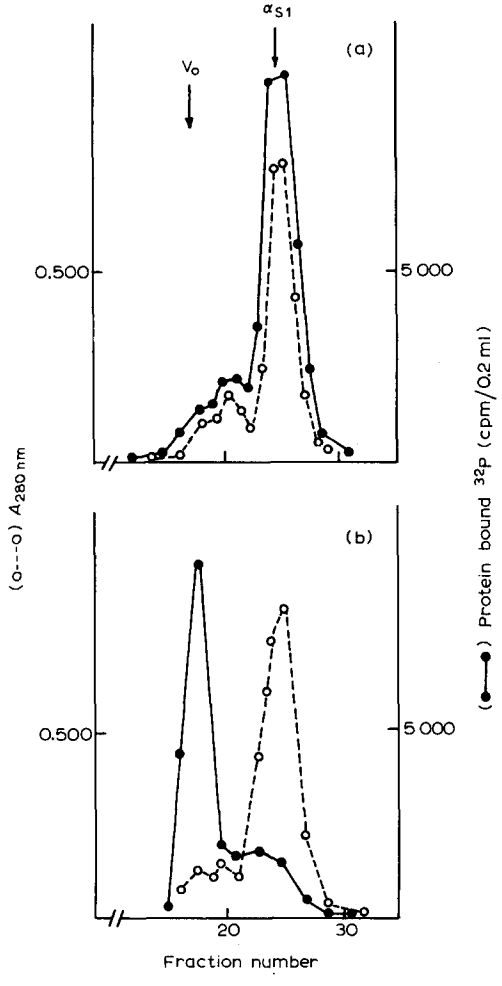

Fig. 7. Sephadex G75 chromatography of α_1-[^{32}P]casein phosphorylated by casein kinase TS (upper figure) and S (lower figure). 15 mg of α_1-casein (Merck) were labeled by [γ-^{32}P]ATP in the presence of casein kinases following the same procedure as described for whole casein under Experimental Procedures, before being submitted to gel filtration in 15% formic acid.

main peak were improved by 0.5% mercaptoethanol. Upon further addition of 2 mM $MgCl_2$ (expected to minimize the contribution of phosphate groups to electrophoretic mobility) the mobility of the radioactive peak remained lower than that of the α_{s1}-casein band.

The above experiments indicate that α_{s1}-casein was significantly phosphorylated only by the TS-form of the enzyme, while some minor casein subfractions other than α_{s1}-casein are phosphorylated by both the TS- and S-forms.

Such a discrimination becomes much more evident when commercial α-casein preparations, instead of whole casein, are submitted to phosphorylation by the two enzymes: although such α-casein preparations are labeled by both the TS- and S-forms, the radioactivity incorporated by the former was actually accounted for by α_{s1}-casein itself (Fig. 7a), while that incorporated in the presence of the S-form was due to contaminations which were completely removed from the α_{s1}-casein absorbance peak by Sephadex G75 gel filtration (fig. 7b) and by polyacrylamide gel electrophoresis (unpublished data).

Discussion

P content, chemical composition and primary structure have already been elucidated for the main casein fractions α_{s1} [10] β and γ- [11] and κ-casein [12].

By our procedure we have shown the preferential involvement in the kinase reaction of two protein fractions separated by DEAE-cellulose column chromatography. These were a less retarded fraction accounting for most of [^{32}P]Thr residues labeled by the TS-form of casein kinase, and almost unaffected by the S-form and a more retarded fraction, characterized by a large predominance of [^{32}P]Ser residues, apparently phosphorylated by both enzymes and clearly heterogeneous. The further purification of these two main fractions by gel filtration and polyacrylamide gel electrophoresis, while confirming the substantial homogeneity of the TR fraction, disclosed the heterogeneity of the SR fraction. Purification studies also suggested that, in spite of their similar behaviour on DEAE-cellulose, the subfractions phosphorylated at serine residues by the two enzymes are not exactly the same. In particular, this is true for the major casein fraction, i.e. α_{s1}-casein, which is phosphorylated by the TS-form, but not significantly by the S-form.

We can conclude from these experiments that by far most of the radioactivity incorporated into whole casein by both casein kinases used in our work is accounted for by minor casein fractions which we can tentatively ascribe to the α_s family according to their chromatographic and electrophoretic behaviour. In particular: (1) All the [^{32}P]Thr residues phosphorylated by the kinase TS appear to be concentrated in a single minor fraction running slightly ahead of β-casein on gel electrophoresis (Fig. 4), like the so called "Fraction 0.86" described by Wake and Baldwin [7]. However, in the light of the recent report that the variant B of β-casein is phosphorylated at much greater rate than other caseins by muscle cyclic AMP-dependent protein kinase [13], the possibility should be carefully considered that our TR fraction might be identified with such a variant of β-casein or with some other variant of the main caseins. (2) Most of [^{32}P]Ser residues labeled by the kinase TS (Fig. 5a) and almost all the

[^{32}P]Ser residues labeled by the kinase S (fig. 5b) are located in other minor casein fractions eluted at the V_0 by Sephadex G75 gel filtration and slightly more retarded than α_{s1}-casein in gel electrophoresis, i.e. behaving like those minor fractions of the α_s-group [14] formerly classified as α_{s2-s5} caseins but consisting, according to Brignon et al. [15], of differently phosphorylated forms of the same α_{s2}-casein. Such an identification is confirmed by the finding that α_{s2}-casein (purified by the method of Brignon et al. [15]) is indeed phosphorylated much more actively than α_{s1}-casein (unpublished data). (3) Among the main casein fractions the only one of those actively labeled appears to be α_{s1}-casein, accounting for some 20—30% of [^{32}P]Ser residues labeled by the kinase TS (Fig. 5a).

It is possible that also the minor radioactivity peak running ahead of α_{s1}-casein during gel electrophoresis might be due to further phosphorylation of α_{s1}-casein itself, through the same (or similar) mechanism as gives rise to α_{s0}-casein, a phosphorylated homologue of α_{s1}-casein [17].

Such general conclusions do not rule out the possibility that the remaining casein fractions undergo a limited phosphorylation by the two enzymes. Low, but significant, amounts of radioactivity are associated with the absorbance peaks of β- and κ-caseins eluted from DEAE-cellulose. However, their labeling is negligible compared with that of the two main radioactive peaks (TR and SR fractions). In a previous paper [2], we reported a very active phosphorylation of κ-casein preparations by both forms of casein kinase, whereas our present data would rule out any significant labeling of κ-casein in whole casein phosphorylated by both enzymes. Such a discrepancy is probably accounted for by the presence of phosphorylatable contaminants in the κ-casein (prepared by the procedure of Zittle and Custer [16]) employed in our previous work; we now have chromatographic evidence that most of ^{32}P incorporated into this fraction by casein kinase TS is bound to minor components rather than to κ-casein, itself.

The heterogeneity of whole casein probably accounts for its wide and successful employment as a substrate for different classes of protein kinase, including both cyclic AMP-insensitive "phosvitin kinases" [1] and "histone kinases" sensitive to cyclic AMP [13], each of which probably can recognize its "right" substrate(s) among the several fractions available in the whole casein. In this respect, the further characterization of the fractions phosphorylated by rat liver "casein kinases" and the identification of the phosphorylation site(s) within their primary structure(s) might provide a critical aid in the understanding of the substrate specificity and the catalytic mechanism of these enzymes, whose biological role is still unknown.

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