STUDIES ON THE STRUCTURAL REQUIREMENTS OF A MICROSOMAL cAMP-INDEPENDENT PROTEIN KINASE

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1. Introduction

The phosphorylation of critical seryl and threonyl residues by specific protein kinases is turning out to be the probably most frequent device by which the biological activity of proteins can be modified. On this matter a crucial problem concerns the understanding of the structural features determining the suitability of just one or a few residues of the target substrate(s) to a definite protein kinase. Such an investigation has been undertaken with the cAMP-dependent protein kinase [1–3], phosphorylase kinase [4] and, recently, in our laboratory also with a cAMP-independent rat liver cytosolic protein kinase (casein kinase TS) that displays a remarkable specificity for the same sites of casein fractions as are phosphorylated endogenously by the mammary gland casein kinase(s) [5,6]. However a second cAMP-independent protein kinase active on casein has been also described in rat liver, referred to as 'casein kinase S' which, unlike casein kinase TS, is mainly present in membraneous structures such as mitochondria [7] and microsomes [8]. This describes the site specificity of such a microsomal cAMP-independent protein kinase as determined by using casein fractions as model substrates, and shows that it displays structural requirements which are different from those of the protein kinases mentioned above.

2. Experimental

Most of the techniques used in the present study (enzymatic phosphorylation and dephosphorylation of casein fractions, BrCN and tryptic digestions, gel filtrations, paper electrophoresis and isolation of ^{32}P-labeled phosphoamino acids and $(Ser{-}P)_n$ clusters) were either described or quoted in [5]. Purified bovine casein fractions were gifts from Dr B. Ribadeau-Dumas. Chymotryptic digestion was performed as in [9].

Casein kinase S was extracted from rat liver microsomes as in [10] and purified by submitting the extract to combined Sepharose 6B gel filtration and phosphocellulose column chromatography, following exactly the same procedure employed for the purification of the cytosolic casein kinase S [11]. The final preparations were constantly completely free of any casein kinase TS and cAMP–dependent protein kinase activity.

3. Results and discussion

3.1. *Substrate specificity of microsomal protein kinase*

As shown in table 1 the microsomal casein kinase S displays a substrate specificity narrower than that of the cytosolic casein kinase TS. In particular, taking into account that after polyacrylamide gel electrophoresis a large part of the radioactivity incorporated by this enzyme into α_{s1}-casein preparations is actually accounted for by other contaminating casein fractions, the corrected value for α_{s1}-casein phosphorylation is almost negligible. Therefore the only two substrates appreciably affected by the microsomal casein kinase S, besides phosvitin which was not used in the present study, are α_{s2}- and β-caseins, both phosphorylated exclusively at seryl residues.

Table 1
Substrate specificity of rat liver casein kinases

Substrate	^{32}P incorporated (cpm) in the presence of	
	Microsomal casein kinase S	Cytosolic casein kinase TS
α_{s1}-casein B	5348	18 251
β-casein A^2	133 715	102 426
α_{s2}-casein	98 886	80 015
κ-casein	1234	1692
Histone H1[a]	1162	5836
BBI[b]	725	16 537

[a] Prepared according to [12]
[b] Soybean Bowman-Birk trypsin inhibitor, prepared according to [13]

Substrate concentration: 2 mg/ml. Incubation time: 30 min. Values are corrected for the radioactivity due to contaminating proteins, removed by polyacrylamide gel electrophoresis. Radioactive contaminants were not detectable in ^{32}P-labeled β- and α_{s2}-caseins

3.2. *Identification of phosphorylation sites in α_{s2}- and β-caseins*

Two seryl residues of α_{s2}-casein become labeled upon incubation with the microsomal protein kinase: they are located within the CNBr fragments 5–26 and 27–141, respectively. The former was identified with Ser_{13} in [5]: indeed, Ser_{13} was found to be phosphorylated also by the cytosolic casein kinase S thus supporting the view that these two enzymes are strictly related to each other, even if not identical.

The identification of the seryl residue(s) phosphorylated within the large CNBr fragment 27–141 turned out to be more difficult since its phosphorylation apparently causes an incomplete tryptic digestion of the labeled CNBr peptide. However by submitting to chymotryptic digestion the CNBr fragment 27–141 labeled by both the microsomal protein kinase and the cytosolic casein kinase TS (at Thr_{130} [5]) it was constantly found that the radioactivity incorporated by the former was completely accounted for by the same chymotryptic peptide also including the [^{32}P]Thr_{130} phosphorylated by casein kinase TS. Thus the residue affected by the microsomal casein kinase S was restricted to the only 3 seryl residues 129, 131 and 135. The first two however can be ruled out for two reasons:

(1) They are already phosphorylated in the native casein and are therefore not available for the kinase;
(2) They are not adjacent to any residue susceptible to tryptic digestion, while once phosphorylated, Ser_{135} would be expected to prevent the tryptic breakdown at the adjacent Lys_{136} and Lys_{137} residues, giving rise to the not predicted tryptic fragment 126–141 whose size exactly corresponds to that of the irregular radioactive tryptic peptide actually observed*.

The phosphorylation of Ser_{135} has been further confirmed by the experiments with definite tryptic peptides described below (see next section).

The identification of the only seryl residue labeled in β-casein was accomplished by submitting the radioactive protein to tryptic digestion after the lysyl residues had been blocked by carbamylation or by maleylation. Only 4 tryptic fragments are obtained by such a procedure which are quite well separated by Sephadex G-50 gel chromatography in 15% formic acid. The whole radioactivity was recovered in the very acidic phosphopeptide 1–25 which also accounts for most of the endogenous unlabeled phosphate of β-casein. Such an identification has been confirmed by paper electrophoresis. Moreover by submitting such a radioactive phosphopeptide to the 12 N HCl hydrolysis procedure [14], suitable for the isolation of the $(Ser–P)_n$ clusters from caseins, it was ruled out that the radioactive residue belongs to the $(Ser–P)_{17–19}$ cluster itself. This finding together with the isolation from the 12 N HCl hydrolysate of small radioactive fragments including also arginine, but not the (Ser–P) cluster, allowed the definite identification of the residue affected by our enzyme with Ser_{22}.

The above results are summarized in fig.1 in which the amino acid sequences including the 3 sites phosphorylated in α_{s2}- and β-caseins are shown. It is evident that the most remarkable common feature of all 3 of them is the presence of a large and very acidic

* Similarly it has been reported that the phosphorylation of Ser_{41} occurring in α_{s0}-casein prevents trypsin from acting on the Lys_{42} residue [14]. By the way the same residue Ser_{41} is very likely to account also for the low radioactivity incorporated into α_{s1}-casein by our microsomal enzyme: such a residue however has to be considered a poor site for phosphorylation as compared with the 3 sites affected in α_{s2}- and β-caseins

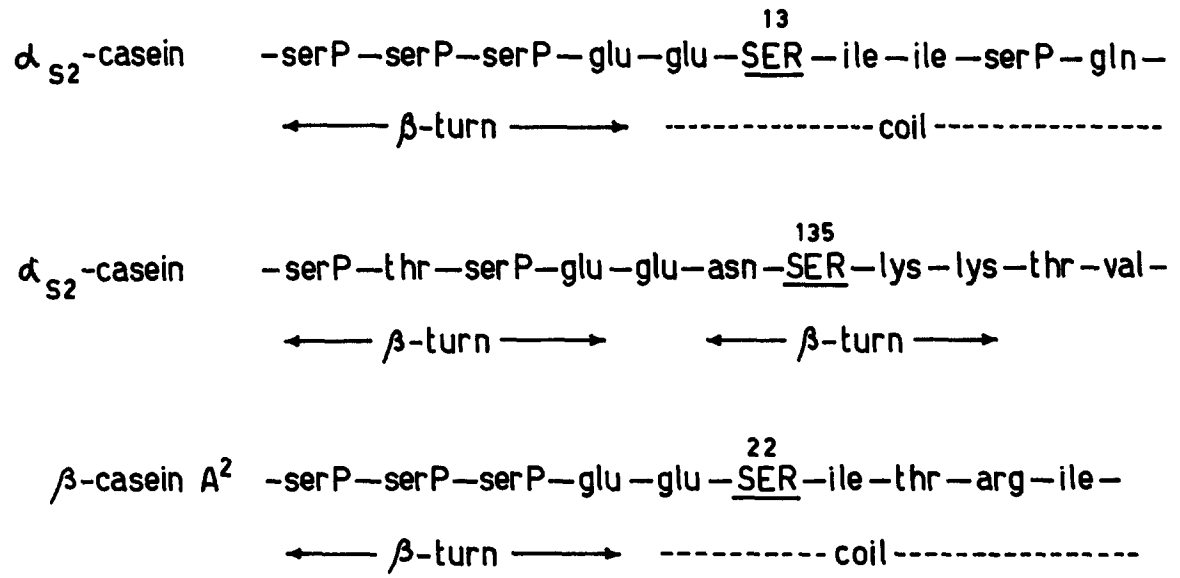

Fig.1. Amino acid sequences at the phosphorylation sites of microsomal casein kinase S in α_{s2}- and β-caseins. The primary structures of α_{s2}- and β-caseins were drawn from [16] and [17], respectively. The empirical predictive model of Chou and Fasman [18] along with their latest conformational parameters [19] were used to analyze for β-turns in the vicinity of the phosphorylation sites, as described [6].

cluster including both glutamyl and phosphoseryl residues close to their N-terminal side. Noteworthy in this connexion is the failure of the microsomal casein kinase S to phosphorylate at appreciable rates κ-casein which lacks such clusters and also α_{s1}-casein which, though containing a $(Ser{-}P)_3{-}(Glu)_2$ sequence identical to those of α_{s2}- and β-caseins lacks any phosphorylatable ser residue near to it on its C-terminal side.

As for the predicted secondary structure of the phosphorylation sites we have calculated that only Ser_{135} of α_{s2}-casein is likely to be located within a β-turn, while the other two residues are not. All three of them however are located just after the end of a predicted β-turn, roughly corresponding to the acidic cluster. It should be recalled that, on the contrary, most of the residues phosphorylated by the cAMP-dependent protein kinase [20] and by the cAMP-independent cytosolic casein kinase TS [6], are actually located within predicted β-turns.

3.3. *Phosphorylation of fragments including the phosphorylatable sites*

In order to obtain further information about the minimum requirements of our microsomal protein kinase, its activity has been tested also toward fragments obtained by CNBr and tryptic digestion that still include suitable sites. As shown in table 2 the phosphorylation rates of most of such peptides, of 16–25 amino acids, is comparable with, and sometimes significantly higher than, that of the intact protein, indicating that the integrity of the protein substrate is not required for optimal activity. This does not mean however that the rest of the molecule does not influence the phosphorylation process: actually as is also shown in table 2, the carbamylation of the lysyl residues of β-casein prevents the phosphorylation of Ser_{22}, though no lysyl residue is directly involved in the phosphorylation process as is confirmed by the finding that the fragment 1–25, once excised by tryptic digestion from the inactive carbamylated β-casein, was found to undergo phosphorylation at the usual rate.

Moreover in order to test the possible role in the kinase reaction of the amino acid sequence C-terminal to the target seryl residue we have determined the phosphorylation rates before and after the peptide chain had been shortened from its C-terminus. As also

Table 2
Labeling rates of phosphorylation sites before and after the fragmentation and/or the modification of the casein molecules

Phosphorylation site	Substrate	Phosphorylation rate (cpm incorporated/10 min)
Ser_{13}	α_{s2}-casein	31 562
	Fragment 5–26[a]	51 724
	Fragment 5–21[b]	40 100
	Fragment 5–21 dephosphorylated	22 380
	Fragment 5–21 methylamine-treated	1043
Ser_{135}	*S*-CM-α_{s2}-casein	35 168
	Fragment 126–141[c]	41 863
	Fragment 126–136 (137)[d]	6310
Ser_{22}	β-casein	83 464
	Fragment 1–25[e]	72 412
	Fragment 1–24[f]	88 250
	Carbamylated β-casein	8982
	Fragment 1–25 from carbamylated β-casein	76 576

[a] Isolated after CNBr digestion of α_{s2}-casein [5]
[b] Prepared by tryptic digestion of fragment 5–26
[c] Isolated after tryptic digestion of the maleylated CNBr peptide 27–141 from *S*-carboxymethylated α_{s2}-casein [5,15]. Tested after demaleylation
[d] Prepared by tryptic digestion of the demaleylated 126–141 fragment
[e] Isolated by Sephadex G-50 gel filtration after tryptic digestion of the maleylated β-casein
[f] Obtained by carboxypeptidase B treatment of the fragment 1–25

Maleylation and demaleylation were performed according to [21]. Carbamylation of β-casein was performed according to [22]. Partial enzymatic dephosphorylation of the fragment 5–25 was accomplished by preincubation with potato acid phosphatase (Boehringer grade II), the phosphatase being inactivated by a short boiling before starting the protein kinase reaction. The conversion of the Ser–P residues into β-methylaminoalanyl residues by methylamine treatment was performed according to [14]. In all the experiments the same molar concentration of substrates was used (1×10^{-4} M)

shown in table 2 the splitting off by carboxypeptidase B of the C-terminal Arg residue from the β-casein phosphopeptide ending with: $\overset{22}{Ser}$–Ile–Thr–Arg, has no negative, but actually a positive effect on the phosphorylation rate of the seryl residue (compare the phosphorylations of fragments 1–25 and 1–24). A basic residue near the C-terminal side of the target one is not therefore required for the kinase reaction. On the other hand the tryptic detachment of the tetrapeptide: –Thr–Val–Asp–Met from the C-terminal part of the α_{s2}-casein fragment 126–141, leaving a peptide ending with $-\overset{135}{Ser}$–Lys–(Lys) causes a remarkable decrease of the phosphorylation rate (compare the phosphorylations of fragments 126–141 and 126–136). Probably therefore also some residues close to the C-terminal side of the target one, besides the acidic cluster on its N-terminal side, play an important role in the site recognition by the protein kinase. In particular the presence of Ile residues adjacent to the C-terminal side of Ser_{22} of β-casein and of Ser_{13} of α_{s2}-casein and that of a Val residue in the small fragment 138–141, whose removal slows down the phosphorylation of Ser_{135}, might suggest that an hydrophobic residue near to the C-terminal side of the target one represents an additional requirement for optimal phosphorylation.

Finally it should be mentioned that an extensive enzymatic dephosphorylation of the $(Ser–P)_{8–10}$ cluster of α_{s2}-casein depresses the phosphorylation

of Ser_{13} while the methylamine-induced conversion of Ser-P into neutral methylaminoalanyl residues completely prevents the kinase reaction. These findings might suggest that the phosphoseryl residues are more important than the glutamyl ones for determining the site recognition by our enzyme and it would also account for the observed failure of the microsomal casein kinase S to phosphorylate the soybean Bowman-Birk trypsin inhibitor (BBI) in spite of the presence in it of the sequence Asp–Asp–Glu–Ser–. The microsomal casein kinase S therefore would not be a 'primary' kinase since it would require for its activity the previous phosphorylation of the substrate, presumably mediated by a second protein kinase. It is also possible however that the negative effect of previous dephosphorylation is due to conformational modifications in the critical acidic cluster region rather than to a specific recognition of the phosphate groups by the kinase.

In conclusion the cAMP-independent microsomal casein kinase S displays a site specificity different from those reported for cAMP-dependent protein kinase [1–3], phosphorylase kinase [4] and cytosolic casein kinase TS which last in turn displays a specificity similar to that of the mammary gland casein kinase [5,6]. Moreover, unlike other protein kinases our enzyme does not apparently display any preferential activity toward residues included within predicted β-turns, but possibly requires residues located after the C-end of β-turns. Its most critical requirement however appears to be the presence of a very acidic cluster close to the N-terminal side of the target seryl residue while an hydrophobic residue on the opposite side is also likely to cooperate in determining the site specificity. Finally, unlike the cytosolic casein kinase TS, the microsomal casein kinase S does not require for its maximal activity the integrity of the substrate protein, although the conformational features of this latter are important for rendering the phosphorylatable sites more or less available to the enzyme.

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