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STRUCTURAL FEATURES DETERMINING THE SITE SPECIFICITY OF A RAT LIVER cAMP-INDEPENDENT PROTEIN KINASE

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SUMMARY: The Seryl and Threonyl residues affected in α_{s1} and in β-caseins by rat liver "casein kinase TS" (a cytosolic cAMP-independent protein kinase) have been identified. All of them, as well as the residues affected by the same enzyme in α_{s2}-casein are characterized by an acidic group two residues to their C terminus and by being located within predicted β-turns. Several other potential sites of phosphorylation, according to their primary structure, but located outside predicted β-turns, are not significantly labeled by the protein kinase. It seems conceivable therefore that both a definite aminoacid sequence including a critical acidic residue, and the existence of a β-turn are required for the activity of this protein kinase.

INTRODUCTION

A very crucial question about Protein kinases is how can these enzymes recognize the "right" residue(s) of the "right" substrate(s). The inspection of many sites affected by the cAMP-dependent Protein kinase in several different substrates (1,2) and studies on the phosphorylation of genetic variants of β-casein (3) and of syntetic peptides (4,5) led to postulate that the presence of two adjacent basic aminoacids, at least one of which is arginine, at a position between the 2nd and the 5th to the N terminal side of the target residue must represent the minimum requirement for the activity of this kinase (6). Moreover it has been recently calculated that most of such phosphorylated residues are located in regions of the proteins predicted as β-turns (7).

On the other hand little was known about the structural requirements of the cAMP-independent Protein kinases. An important exception was the mammary gland caseinkinase, supposed to require an acidic group two residues toward the C terminus of the target one, since all the phosphorylserine residues found in native caseins apparently fulfill such a rule (8,9).

Recently however we have presented evidences suggesting that a rat liver cAMP-independent Protein kinase, the so called "caseinkinase TS" (10), displays a site specificity similar if not identical to that of mammary gland caseinkinase since it phosphorylates residues of α_{s2}-casein which either are also phosphorylated in the native protein or can be considered potential sites of phosphorylation for the latter enzyme (11). The data presented in this paper, obtained using α_{s1}- and β-caseins as model substrates for the proteinkinase TS, provide the definite evidence that such an enzyme affects both seryl and threonyl

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residues having an acidic group two residues to their terminal side. Moreover a comparative analysis of the sites which are actually phosphorylated and of other potential sites of phosphorylation which however are not affected by the enzyme, discloses a remarkable parallelism between the susceptibility to the kinase and the location within a predicted β-turn, thus suggesting that such a structural feature may play, also in the case of the cAMP-independent proteinkinases, an important role in optimizing the availability to the enzyme of the suitable sites.

EXPERIMENTAL

Most of the techniques used in the present study (purification of Proteinkinase TS, enzymatic phosphorylation and dephosphorylation of casein fractions, BrCN and tryptic digestions, gel filtrations, paper electrophoresis and isolation of ^{32}P-labeled phosphoserine, phosphothreonine and $(ser\text{-}P)_n$ clusters) were either described or quoted in a previous paper (11). Purified bovine caseins $\alpha_{s1}B$ and βA^2, prepared according to Mercier et al. (12) were gifts from Dr. B.Ribadeau-Dumas. Both Ser and Thr residues were found to be labeled in α_{s1}-casein, to give a Thr-^{32}P/Ser-^{32}P ratio approaching 1.3, while virtually all the ^{32}P incorporated into β-casein was accounted for by Thr-^{32}P.

The empirical predictive model of Chou and Fasman (13) along with their latest conformational parameters (14) were used to analyze for β-turns in the vicinity of the phosphorylated sites and of potential sites of phosphorylation, the approach being very similar to that applied to other phosphorylated protein sites by Small et al. (7).

RESULTS AND DISCUSSION

The isolation of radioactive fragments from the ^{32}P labeled α_{s1}-casein is outlined in Fig.1. As shown in Fig. 1A both ser-^{32}P and thr-^{32}P residues are confined only to peptide Tm1 obtained by tryptic digestion of the maleylated protein and which is quite well separable from the other peptides by paper chromatography (15). Once the radioactive peptide Tm1, corresponding to the sequence 23-90, is submitted again to tryptic digestion after demaleylation, it splits into two radioactive fragments, separable by Sephadex G50 gel chromatography (Fig. 1B). The less retarded fragment contained the ser-^{32}P and the more retarded one the thr-^{32}P. The thr-^{32}P containing peptide has been identified with the expected tryptic fragment 43-58, according to its size, electrophoretic mobility and aminoacid composition. The only thr residue, which is therefore necessarily also the only thr residue phosphorylated in α_{s1}-casein by protein kinase TS, is thr_{49}, which thus represents a potential site of phosphorylation for the physiological caseinkinase due to the acidic residue Asp_{51}. The Ser-^{32}P containing peptide has been identified with the largest expected tryptic fragment 59-79. By concentrated HCl hydrolysis followed by pH 1.5 paper electrophoresis-i.e. the Williams and Sanger procedure to isolate $(Ser\text{-}P)_n$ clus-

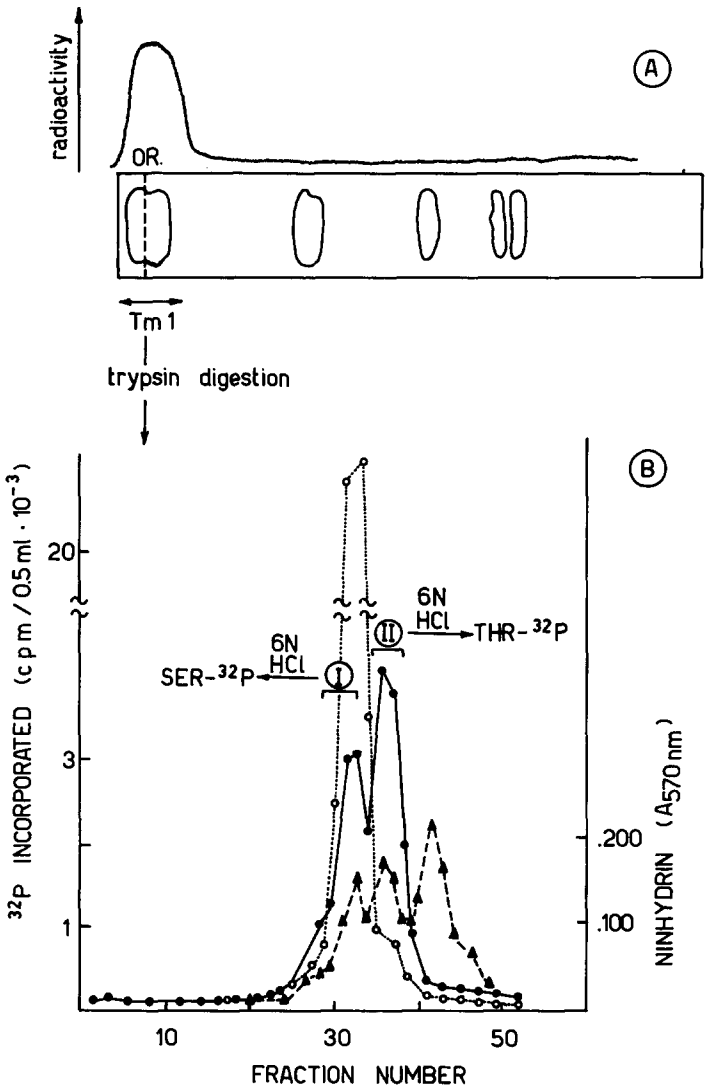

Fig. 1 - Isolation of radioactive fragments from ^{32}P-α_{s_1}-casein labeled by caseinkinase TS.

A) 8 mg of labeled casein maleylated and digested with trypsin (16) was submitted to 15 hrs ascending paper chromatography (eluent Buthanol-Pyridine--Acetic acid-water, 120-80-24-96) after its demaleylation with 30% acetic acid. The radioactivity was localised by a chromatoscanner and peptides detected by the ninhydrin reaction on a parallel strip.
B) The radioactive demaleylated peptide Tm1 eluted from A was further digested with trypsin (50 µg) for three hrs at 37° in 0.25 ml of 0.1 M NH_4HCO_3 pH 7.8--8.0. The digested sample was made 15% with formic acid and submitted to Sephadex G50 gel chromatography following a procedure previously described (11). Radioactive profiles: ●——●, peptides from native ^{32}P-casein; o·····o peptides from ^{32}P-casein previously 50% dephosphorylated with acid phosphatase (8). The ninhydrin profile (▲-----▲) was obtained after alkaline hydrolysis (20) of 0.5 ml aliquots previously dried at 105°. Fractions I and II, collected as indicated, were analyzed for their aminoacid composition and contents of radioactive phosphoserine, phosphothreonine and phosphoserine clusters.

ters (16) - it was possible to localize the labeled residue(s) within the sequence 66-68 which corresponds to the only $(Ser\text{-}P)_3$ cluster of native α_{s1}--casein. The labeling of such a $(Ser\text{-}P)_3$ block by proteinkinase TS is probably due to an incomplete endogenous phosphorylation of it in at least few molecules of native α_{s1}-casein. In accordance with this hypothesis the prior partial dephosphorylation of the casein by acid phosphatase dramatically increases the susceptibility of the sequence 66-68, and therefore of the tryptic fragment 59-79, to the proteinkinase, while the labeling of Thr_{49}, under these conditions, drops to neglegible values (dotted line of Fig. 1B). This suggests that the Thr residues are phosphorylated only when suitable Ser residues are not available.

The identification of the Thr residue of β-casein A^2 undergoing phosphorylation was accomplishes by a similar procedure: ^{32}P-β-casein was first submitted to BrCN digestion followed by Sephadex G50 gel chromatography. All the radioactivity was recovered in the largest peptide, corresponding to the fragment 1-93. Such a fragment was digested with trypsin and submitted again to Sephadex G50 gel chromatography: more than 90% of the radioactivity was now recovered in a single peptide with a M.W. of about 2,000 and identified with the fragment 32-48, containing only one Thr residue, which must be therefore also the only one phosphorylated at the 41 position (17).

The above results are in agreement with those obtained with α_{s2}-casein (11) and together they strongly support the conclusion that the rat liver protein kinase TS requires the same structural features in the substrate as the physiological caseinkinase. In fact, as shown in Table I, all the six sites affected by the liver protein kinase in α_{s1}-, β- and α_{s2}-caseins are either Ser residues belonging to the $(Ser\text{-}P)_3$ clusters also phosphorylated <u>in vivo</u> or Thr residues which are to be considered potential sites of endogenous phosphorylation by virtue of having an acidic group two residues toward the C terminus.

Surprisingly however several other potential sites of phosphorylation according to this rule, also listed in Table I, are not apparently affected by Proteinkinase TS. Such a finding and the recent report that many phosphorylated residues are located within predicted β-turns (7) prompted us to look for the probability of occurrence of β-turns around the residues actually phosphorylated and those which are not in spite of being potential sites of phosphorylation. The results of such an analysis are also reported in Table I: they show that all the six phosphorylated residues, assuming a cut-off value of p_t=0.50, or at least five of them, if we assume the higher value of p_t=0.75, are indeed located within regions predicted as β-turns. On the other hand 5 out of 7 residues which are not phosphorylated though representing potential sites of phosphorylation are within regions not predicted as β-turns for their

TABLE I - A comparison between the sites of casein fractions which are phosphorylated by rat liver proteinkinase TS and other potential sites of phosphorylation.

Casein substrate	Sites actually labeled by liver proteinkinase +	p_t value (10^{-4}) *	Predicted as β-turn
αs_1	[-$\overset{49}{\text{THR}}$-glu-asp-gln-]	0.90	YES
β	[-$\overset{41}{\text{THR}}$-glu-asp-glu-]	0.59	YES/NO ‡
α_{s2}	[-ser-$\overset{130}{\text{THR}}$-ser-glu-]	1.03	YES
α_{s1}	[$(\overset{66-68}{\text{SER}})_3$-glu-glu-ile ++	1.33	YES
α_{s2}	[$(\overset{8-10}{\text{SER}})_3$-glu-glu-ser- ++	1.33	YES
α_{s2}	[$(\overset{56-58}{\text{SER}})_3$-glu-glu-ser- ++	1.33	YES
	Potential sites of phosphorylation NOT labeled by liver prot.kinase		
α_{s1}	[-$\overset{41}{\text{SER}}$-lys-asp-ile-] **	1.30	YES
α_{s2}	[-$\overset{3}{\text{THR}}$-met-glu-his-]	0.29	NO
α_{s2}	[-$\overset{31}{\text{SER}}$-lys-glu-asn-] **	0.96	YES
α_{s2}	[-ala-$\overset{66}{\text{THR}}$-val-asp-]	0.31	NO
α_{s2}	[-ile-$\overset{72}{\text{THR}}$-val-asp-]	0.14	NO
α_{s2}	[-$\overset{138}{\text{THR}}$-val-asp-met-]	0.40	NO
α_{s2}	[-leu-$\overset{154}{\text{THR}}$-glu-glu-]	0.32	NO

+ The residues actually or potentially phosphorylatable are in CAPITALS and numbered. The critical acidic residues expected to determine the phosphorylation are underlined. Brackets include either the predicted β-turns or anyway the tetrapeptides containing both the phosphorylatable and the critical acidic residues which display the highest p_t values

* Calculated as described in the experimental section for the corresponding tetrapeptides (between brackets).

‡ Depending on the cut-off value assumed (see text).

++ The labeling of such $(ser\text{-}P)_3$ clusters (also phosphorylated in vivo) by the liver proteinkinase is greatly enhanced by previous dephosphorylation. The number and identity of the single residue(s) actually phosphorylated within the clusters are unknown.

** These are probably not efficient sites of phosphorylation for the presence of a lys residue (see text).

low p_t values. Moreover it should be noted that the two only exceptions, namely ser_{31} of α_{s2}-casein and ser_{41} of α_{s1}-casein, are both characterized by the unique presence of a lys residue between them and the acidic residues supposed to determine the specificity of the enzyme; therefore assuming that a negative charge near to the C terminus of the target residue is required for the acti-

vity of the kinase (18) it is quite doubtful that such two residues still represent powerful sites of phosphorylation. Such a conclusion is also in agreement with the report that Ser_{41} *in vivo* undergoes a very limited phosphorylation, compared with the nearly complete phosphorylation of the other phosphoseryl residues of α_{s1}-casein, giving rise to the conversion of only few molecules of α_{s1}-casein into so called α_{s0}-casein (9). Therefore, if we calcel from the list of potential sites of phosphorylation the two ser having an adjacent lys residue, all the remaining potential sites not phosphorylated by our kinase are located outside predicted β-turns.

In conclusion the results reported in this paper provide the definite evidence, based on the phosphorylation of three different substrates, that, as previously suggested (11), the rat liver proteinkinase TS displays a site specificity similar to that of the mammary gland caseinkinase, since it requires an acidic group (an asp being apparently as efficient as a glu) two residues toward the C terminus of the target residue, which can be either Ser or Thr. The presence of an adjacent basic residue seems to prevent or reduce the efficiency of the acidic one. However such a minimum requirement resting on the aminoacid sequence, apparently is not a sufficient condition for the phosphorylation to occur, since several potential sites of phosphorylation though fulfilling it, are not affected by the kinase: likely the conformation of the protein substrate may play a crucial role in rendering the suitable residues more or less available to the enzyme, and it is remarkable on this matter the rather close parallelism between phosphorylability and location within β-turns. This finding is in good agreement with the previous reports that also the sites phosphorylated by other protein kinases, displaying site specificity different from ours, are frequently within β-turns (7) and that ser_{149} and ser_{151} which are found phosphorylated in native cow and sheep caseins are also included in predicted β-turns (20). It is conceivable therefore that such a conformational feature might indeed represent a general condition for the phosphorylation of proteins.

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