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SYNTHETIC FRAGMENTS OF PROTAMINES AS MODEL SUBSTRATES FOR RAT LIVER CYCLIC AMP-DEPENDENT PROTEIN KINASE *

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Nine synthetic peptides reproducing either exactly or with suitable substitutions three phosphorylatable sites of the protamines thynnine Z1 and galline have been prepared and tested as phosphate acceptors for the rat liver cyclic AMP-dependent protein kinase (type I). The most significant results obtained can be summarized as follows: 1. The hexapeptide: Arg-Arg-Ser-Thr-Val-Ala gives two phpsphorylated products, containing only Ser-P and both Ser-P and Thr-P, respectively. The relative amount of the di-phosphorylated derivative is not dependent on the incubation time but rather on the concentration of the substrates. 2. Both the replacements of ornithine for the two N-terminal arginines of glutamic acid for Val_5 in the above peptide completely prevent phosphorylation, without conferring any inhibitory activity to the modified peptides, thus supporting the view that the N-terminal guanido groups and the C-terminal hydrophobic residue(s) are both required for the binding at the catalytic site rather than for the subsequent transphosphorylation reaction. 3. The replacement of alanine for Ser_3 gives rise to a peptide whose Thr_4 residue is still phosphorylated with an efficiency comparable to that of the unmodified peptide. The di-substituted peptide: Arg-Arg-Ala-Ser-Val-Ala however exhibits a dramatically lower K_m value indicating that serine is a much better target than threonine whenever it is not adjacent to the N-terminal arginine couple. 4. The importance of the distance between the target residue and the N-terminal basic determinants is also evidenced by the phosphorylation of the dodecapeptide: Pro-$(Arg)_5$-Ser-Ser-Arg-Pro-Val-Arg exhibiting a K_m value about 20-times higher than that of salmine A1, whose phosphorylation site is comprised within an identical amino acid sequence, including however three rather than two adjacent serine residues. 5. The tetradecapeptide: $(Arg)_4$-Tyr-Gly-Ser-$(Arg)_6$-Tyr is completely unaffected by the kinase though a very similar site is found phosphorylated in native iridines, probably through a cyclic AMP-independent mechanism.

Introduction

Among the factors responsible for the specificity of protein kinases the primary structure around the phosphorylation site has been shown to play a relevant role. Evidence has been accumulated that different protein kinases are able to recognize residues of the protein substrates characterized by distinct and definite aminoacid sequences [1–5].

In particular the primary structure requirements of the cyclic AMP-dependent protein kinase have been extensively investigated both by inspecting the aminoacid sequences around the residues phosphorylated in several substrates (reviewed in Ref. 6) and by studying the phosphorylation of simple synthetic

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peptides reproducing with suitable substitutions the structural features which are supposed to be critical for the phosphorylation to occur [7–10].

In brief, it was thus possible to establish that relatively simple penta- to dodeca-peptides still display a phosphorylation efficiency comparable to that of intact protein substrates provided that a very critical couple of basic residues (one of which must constantly be an arginine) are present in the vicinity of the N-terminal side of the target residue [7,8], which apparently also needs to have a hydrophobic group close to its C terminus [7].

Several other local features however, which are also supposed to influence the site specificity of this kinase - like the suitability of threonine instead of serine residues, the number and nature of the residues between the basic and the target ones, the actual importance of the carboxy terminal residues, the availability of adjacent hydroxylic residues - are still either unclarified or under debate.

The presence in protamines of several sites potentially suitable for the cyclic AMP-dependent protein kinase, some of which are actually phosphorylated also in vivo [11,12], prompted us to synthesize peptides reproducing such sites and to test their efficiency as substrates of the cyclic AMP-dependent kinase. A first set of hexapeptides reproduces with suitable substitutions a phosphorylatable site of thynnine Z1 characterized by the presence of the unusual couple Ser-Thr, C terminal to a pair of arginines and followed by two hydrophobic residues. The dodecapeptide Pro-Arg_5-Ser_2-Arg-Pro-Val-Arg corresponding to the second phosphorylatable site of thynnine Z1 was also obtained as well as a tetradecapeptide corresponding to the C terminal fragment of galline, whose serine residue is the N terminal to a cluster of six arginine residues. The phosphorylation of such peptides by rat liver cyclic AMP-dependent protein kinase was studied and compared with that of the phosphorylation site of salmine A1. The kinetic constants were calculated and the phosphorylated residues have been identified. This paper reports on such a study, which, while confirming previous data, provides new information especially about the suitability of threonine residues, the phosphorylation of adjacent residues and other local structural factors influencing the site recognition by the cyclic AMP-dependent protein kinase.

Experimental

Peptide synthesis and purification. The peptides used in this paper were synthesized by the method in solution using the approach of substituting all the arginine residues of the sequence by ornithines and transforming them into arginines by amidination of the final peptides [13].

This approach allows the evaluation of ability of the ornithine analogs to behave as substrates in comparison with the arginine peptides. After complete removal of the protecting groups the amidination reaction was carried out by 1-guanyl-3-5-dimethylpyrazole nitrate [14] and the products were purified by gel filtration on a Sephadex G-10 column. All synthetic peptides were characterized by TLC, gel electrophoresis and amino acid analysis of acid hydrolyzate (Table I). The conversions of ornithine to arginine were generally better than 90%. In some cases a little loss of serine was found.

The synthesis of the tetradecapeptide was previously reported [13]. The pentaptide A_6 was prepared by condensation of tri-carbobenzossi-L-arginine-hydrossisuccinimido ester on the tetrapeptide H-Ser-Thr-Val-Ala-OEt. The product was hydrogenated over black palladium in dimethylacetamide with 20% acetic acid and the final peptide precipitated from ethanol ether was chromatographically pure in TLC.

The details of the synthesis of other peptides will be reported elsewhere. In Table I the amino acid analyses of the acid hydrolysates of peptides are reported.

Enzymatic phosphorylation. The phosphorylation of synthetic peptides was carried out with the cyclic AMP-dependent protein kinase isolated from rat liver according to Titanji et al. [15]. The reaction mixture, with a final volume of 0.25 ml, contained 100 μM [γ-^{32}P]ATP (30–50 cpm/pmol)/100 mM Tris-HCl buffer, pH 7.5/12 mM $MgCl_2$/1 μM cyclic AMP/synthetic peptides (0.5 mg)/cyclic AMP-dependent kinase preparation (5–15 μg protein). In control experiments either the cyclic AMP or the peptides were omitted. Incubations were carried out at 37°C and lasted 15 min (5 min for kinetic experiments) unless differently indicated. Reactions were terminated with 50 μl 0.25 M HCl. The phosphorylated peptides were separated from [γ-^{32}P]ATP by high voltage paper electrophoresis at pH 1.9 (2.5% formic acid plus 7.8% acetic acid) on Whatmann 3-MM paper

TABLE I

AMINO ACID COMPOSITION OF SYNTHETIC PEPTIDES FOLLOWING ACID HYDROLYSIS

The analyses were carried out with a Carlo Erba analyzer mod. 3A28M. Values given are mol amino acid/mol peptide.

Peptides	Thr	Ser	Pro	Glu	Gly	Ala	Val	Tyr	Orn	Arg
a) Z-(Orn(Boc))$_2$-Ser-Thr-Val-Ala-OEt	1.02	0.98	–	–	–	0.99	1.00	–	1.97	–
b) Amidinated derivative	1.00	1.00	–	–	–	0.99	1.00	–	0.17	1.78
a) Z-(Orn(Boc))$_2$-Ser-Thr-Glu-Ala-OEt	1.01	1.01	–	1.04	–	0.99	–	–	1.98	–
b) Amidinated derivative	1.00	0.97	–	1.06	–	0.99	–	–	0.10	1.80
a) Z-(Orn(Boc))$_2$-Ala-Thr-Val-Ala-OEt	1.10					2.00	1.10		1.80	
b) Amidinated derivative	1.06					1.92	1.02		0.20	1.71
a) Z-(Orn(Boc))$_2$-Ala-Ser-Val-Ala-OEt		0.94				2.00	1.00		2.10	
b) Amidinated derivative		0.94				2.07	1.00			1.94
Arg-Ser-Thr-Val-Ala-OEt	1.03	1.06				0.96	0.98			0.95
a) Z-Pro-(Orn(Boc))$_5$-Ser$_2$-Orn(Boc)-Pro-Val-Orn(Boc) · OMe	–	1.98	2.20	–	–	–	1.02	–	7.30	–
b) Amidinated derivative	–	1.50	2.07	–	–	–	1.00	–	0.90	6.60
a) Z-(Orn(Boc))$_4$-Tyr-Gly-Ser-(Orn(Boc))$_6$-Tyr	–	1.00	–	–	0.96	–	–	2.03	10.10	
b) Amidinated derivative	–	0.93	–	–	1.03	–	–	2.04	0.08	10.03

(60 min, 150 V/cm). The electropherograms were scanned with a Packard radiochromatogram scanner: the areas under the radioactive peaks corresponding to the phosphopeptides were cut out and counted in a liquid scintillator.

The phosphorylation of histones and protamines was performed under identical conditions; the reactive proteins were however separated from [γ-^{32}P]-ATP by precipitation with trichloroacetic acid/silicotungstic acid solution as previously described [16].

Isolation and estimation of labeled phosphoaminoacids. ^{32}P-Serine and ^{32}P-threonine were isolated from phosphopeptides by 6 M HCl hydrolysis (4 h at 110°C) followed by pH 1.9 high voltage paper electrophoresis as previously described [17]. The experimental values were corrected for hydrolytic losses of 57 and 18% for Ser-^{32}P and Thr-^{32}P, respectively [18].

Results

The phosphorylation rates of eight synthetic peptides reproducing either exactly (peptides A1, A6 and B) or with suitable substitutions (peptides A2, A3, A4, A4S and A5) the two potentially phosphorylatable sites of thynnine Z1, are reported in Table II. Both sites include a couple of adjacent hydroxylic residues, namely Ser-Thr and Ser-Ser, and both are readily phosphorylated by the cyclic AMP-dependent kinase in the peptides A1 and B, respectively, displaying phosphorylation rates quite comparable with those observed when protein substrates like histones and protamines are used.

The suppression of the serine residue of peptide A1 by replacement with alanine in peptide A4 does not prevent but just slows down the phosphorylation rate, thus indicating that the threonine residue is still a fairly good target for the protein kinase.

However, the substitution of serine for threonine in peptide A4 to give the di-substituted peptide A4S, increases the phosphorylation rate, indicating that serine is a better target than threonine.

On the other hand both the replacements of two ornithines for the pair of N-terminal arginines (in A2 and A5) and of glutamic acid for the valine residue C terminal to threonine (in A3) completely prevent phosphorylation. The resulting peptides are also unable to induce any inhibition on the phosphorylation of A1, suggesting that they have also lost the capability to bind to the catalytic site.

Moreover the pentapeptide A6, lacking the N-terminal arginine does not undergo any more phosphorylation indicating that both arginine residues are critically required by the kinase.

TABLE II

RATE OF PHOSPHORYLATION BY THE CYCLIC AMP-DEPENDENT PROTEIN KINASE OF SYNTHETIC PEPTIDES REPRODUCING FRAGMENTS OF THYNNINE Z1 AND GALLINE

Peptides A1, A6, B and C exactly reproduce the fragments 18-23 and 1-12 of thynnine Z1 and 52-65 of galline, respectively. In the other peptides the substituted residues are underlined. Peptide concentration was 2 mg/ml, unless differently indicated. No detectable phosphorylation of peptides A2, A3, A5 and C could be evidenced even increasing their concentration up to 10 mg/ml.

Peptide	Amino acid sequence	Phosphorylation rate (% of rate with salmine A1)	
		+cyclic AMP	−cyclic AMP
A1	H-Arg-Arg-Ser-Thr-Val-Ala-OEt	223	26
A2	H-Orn-Orn-Ser-Thr-Val-Ala-OEt	<1	<1
A3	H-Arg-Arg-Ser-Thr-Glu-Ala-OEt	<1	<1
A4	H-Arg-Arg-Ala-Thr-Val-Ala-OEt	135	15
A4S	H-Arg-Arg-Ala-Ser-Val-Ala-OEt	348	48
A5	H-Orn-Orn-Ala-Thr-Val-Ala-OEt	<1	<1
A6	H-Arg-Ser-Thr-Val-Ala-OEt	<1	<1
A1 (2 mg/ml) + A2 (8 mg/ml)		226	25
A1 (2 mg/ml) + A3 (8 mg/ml)		227	24
B	Z-Pro-$(Arg)_5$-Ser-Ser-Arg-Pro-Val-Arg-OMe	177	19
C	$H(Arg)_4$-Tyr-Gly-Ser-$(Arg)_6$-Tyr-OH	<1	<1
Histones (Sigma, type IIA)		126	26
Salmine A1		100	20

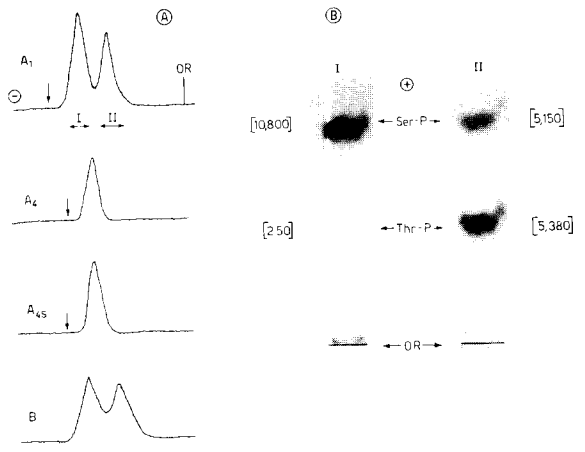

Fig. 1. High voltage paper electrophoresis of ^{32}P-labeled phosphopeptides A1, A4, A4S and B, and isolation of phosphoaminoacids from A1 subfractions. (A) The labeled peptides were electrophoresed at pH 1.9 for 60 min at 160 V/cm and the electrophoretograms were scanned with a Packard radiochromatogram scanner. The arrows denote the distance migrated by the non-phosphorylated peptides. (B) The areas corresponding to the fractions I and II from ^{32}P-labeled A1 were cut out as indicated in A and eluted with 10% acetic acid. The eluates were analyzed for their Ser-^{32}P and Thr-^{32}P content after acid hydrolysis as described in the experimental section. The autoradiographies of the corresponding electrophoretograms are shown. Numbers between brackets refer to the cpm accounted for by Ser-^{32}P and Thr-^{32}P after correction for hydrolytic losses (57 and 18%, respectively) [18].

TABLE III

KINETIC CONSTANTS FOR CYCLIC AMP-DEPENDENT PRCTEIN KINASE WITH SYNTHETIC PEPTIDES AND SALMINE A1

The kinetic constants were determined from initial rate measurements by double-reciprocal plots. The residues undergoing phosphorylation are underlined: dotted underlining denotes minor sites of phosphorylation. The identification of the main phosphorylation site of salmine as Ser_8 is as yet unpublished.

Substrate and amino acid sequence	App. K_m (μM)	V (pmol P/min)
A1: H-Arg-Arg-Ser-Thr-Val-Ala-OEt	2 200	340
A4: H-Arg-Arg-Ala-Thr-Val-Ala-OEt	2 200	213
A4S: H-Arg-Arg-Ala-Ser-Val-Ala-OEt	48	533
B: Z-Pro-$(Arg)_5$-Ser-Ser-Arg-Pro-Val-Arg-OMe	1 100	320
Salmine A1: Pro-$(Arg)_4$-Ser-Ser-Ser-Arg-Pro-Val-Arg	53	250

It should be noted on the other hand that the tetradecapeptide C, reproducing the C-terminal fragment of galline [19] and having six arginine residues but no hydrophobic residues in its C terminal part, is quite unaffected by the kinase in spite of its four arginine cluster separated from the N terminus of serine by two residues, which has been proposed to represent the optimal spatial situation for the phosphorylation of protamines by the cyclic AMP-dependent kinase [6].

The kinetic parameters for protein kinase obtained with the phosphorylatable synthetic peptides A1, A4, A4S and B are reported in Table III. It can be seen that the K_m values with A1 and A4 are identical, the former however being a better substrate thanks to a significantly higher V. On the other hand the apparent K_m value with peptide B is about 20-times greater than that determined with salmine A1, which is especially noticeable considering the striking homologies between the dodecapeptide B and the 12 N-terminal amino acid residues of salmine, including the phosphorylation site (see Table III).

Finally it should be noted that the di-substituted peptide A4S displays by far the lowest K_m value, which fits with the finding that optimal kinetic constants are exhibited by peptides containing one amino acid residue between the basic residues and serine [10].

The finding that the hexapeptide A4, including only a threonine residue, nevertheless undergoes phosphorylation at appreciable rates, raised the question whether the phosphorylation of the hexapeptide A1 involves also its Thr_4 or only its Ser_3 residue. It was firstly ascertained that both Ser-^{32}P and Thr-^{32}P can be isolated from labeled A1 by partial acid hydrolysis. This result however didn't clarify whether serine and threonine are becoming phosphorylated together in the same molecules or independently within distinct peptides. In order to discriminate between these possibilities the phosphorylated peptides were submitted to high voltage paper electrophoresis at pH 1.9: as shown in Fig. 1 while ^{32}P-A4 and ^{32}P-A4S were electrophoretically homogeneous, the phosphorylated A1 was resolved into two radioactive components, I and II, both more acidic than the unphosphorylated A1. Upon elution and partial acid hydrolysis only Ser-^{32}P could be isolated from the component I, while both Ser-^{32}P and Thr-^{32}P in equal amounts (after correction for hydrolytic loss) were isolated from component II (Fig. 1b). Both their electrophoretic mobility and their phosphoamino acid content univocally lead to the identification of the component I as A1 phosphorylated only at its serine residue (SerP-Thr = A1-P) and of the component II as the di-phosphorylated derivative of A1 (SerP-ThrP = A1-P_2). No evidence could be obtained about any formation of A1 phosphorylated only at its threonine but not at its serine residue.

TABLE IV

INFLUENCE OF DIFFERENT EXPERIMENTAL CONDITIONS ON THE FORMATION OF THE MONO- AND DIPHOSPHORYLATED DERIVATIVES OF PEPTIDE A1

Fractions I (A1-^{32}P) and II (A1-$^{32}P_2$) were separated electrophoretically and eluted as described in Fig. 1. Comparable aliquots were counted in a liquid scintillator.

A1 conc. (mg/ml)	ATP conc. (μM)	Incubation time (min)	^{32}P Incorporated (cpm):		A1-P_2/A1-P ratio
			A1-^{32}P	A1-$^{32}P_2$	
0.25	60	15	1 300	1 422	1.09
0.50	60	15	2 551	2 067	0.81
1.00	60	15	5 636	2 700	0.47
2.00	60	15	8 855	3 586	0.40
4.00	60	15	11 687	3 923	0.33
2.00	300	15	9 854	6 602	0.67
2.00	60	5	3 612	1 450	0.40
2.00	60	30	13 886	5 490	0.39

It should be noted that the ratio A1-P_2/A1-P increases while the concentration of A1 in the incubation medium decreases (Table IV), whereas it is apparently independent of the incubation time. High ATP concentrations also favour the formation of A1-P_2.

On the other hand the capability of the rat liver cyclic AMP-dependent protein kinase to phosphorylate two adjacent hydroxylic residues is also confirmed by the finding that the phosphorylation product of peptide B is electrophoretically hetherogeneous (Fig. 1A) suggesting that both the mono- and di-phosphorylated peptides were formed.

Discussion

The phosphorylatable site Ser_{20}-Thr_{21} of thynnine Z1, exactly reproduced in the hexapeptide A1, fulfils both the assessed requirements of the cyclic AMP-dependent protein kinase, having a couple of arginine residues close to its N-terminal side and a hydrophobic residues on its C terminus; accordingly it is a fairly good substrate for the kinase. Our results confirm that both these requirements are absolutely essential: in fact either the substitution of arginine by ornithine, or the replacement of glutamic acid for the C terminal valine completely prevent phosphorylation. Actually the lack of any hydrophobic residue in its C terminal part also probably accounts for the failure of the tetradecapeptide C, reproducing a fragment of galline, to undergo phosphorylation.

The inability of the couple Orn-Orn to successfully replace the couple Arg-Arg points to a specific involvement of the guanido groups, rather than merely of positively charged groups, and fits with the hypothesis that the critical arginine residue, besides being recognized by a complementary acidic residue of the enzyme [20], is also required for stabilizing, through hydrogen bonding, a critical β-bend structure including the target residue(s) [21].

Moreover, the failure of the modified peptides A2 and A3 to inhibit the phosphorylation of the suitable peptide A1, even when added in large molar excess, suggests that both the critical groups, guanido and hydrophobic, are required for the binding rather than for the subsequent catalytic transphosphorylation reaction.

The rather high apparent K_m obtained with peptide A1, compared with that of peptide A4S, is probably a consequence of the contiguity between the two arginine and the serine residues, whereas the optimal structure for phosphorylation has been proposed to be that with either one [10] or two [6] amino acid residues between the basic residues and the serine. The one residue spatial requirement is fulfilled in both peptides A4 and B: yet their phosphorylation efficiency is either lower or only slightly higher than that of A1 (see Table III). In the case of A4 a reasonable explanation might be the presence of threonine which appears to be less suitable than serine for phosphorylation (see below).

The relatively high apparent K_m of the dodecapeptide B on the other hand is possibly accounted for by the presence of a C terminal basic residue, which has been shown to represent a negative factor [8]. Surprisingly however it is also 20-times higher than the K_m of salmine, whose phosphorylation site, included within its N terminal part, displays an amino acid sequence strikingly identical to that of the dodecapeptide B save for the replacement of a serine for an arginine (see Table III). Since it has been shown that the length of the substrate, beyond six to seven residues, is not important for improving the kinetic constants with the cyclic AMP-dependent kinase [7,10] it should be concluded that the sequence Arg-Arg-Ser-Ser-Ser-Arg-Pro of salmine is much more suitable for phosphorylation than the almost identical sequence Arg-Arg-Ser-Ser-Arg-Pro of the dodecapeptide B having two, rather than three consecutive serine residues. Such a conclusion fits very well with the previous results of Shenolikar and Cohen [6] suggesting that the presence of two, rather than only one, residues between the target and the basic amino acids represents the optimal condition for the phosphorylation of clupeines by the cyclic AMP-dependent kinase. However, using a set of dodecapeptides sharply different for amino acid composition from the protamines and the protamine fragments used by us and by Shenolikar and Cohen [6], Fermisco et al. [10] have shown that the best conditions for phosphorylation occur when the phosphorylated residue was separated from the set of arginines by one, not two, amino acid residues. It is therefore likely that the optimal spatial requirements for the location of basic residues are variable depending on other factors. including perhaps the nature of the C terminal residues.

The phosphorylation of threonine residues by the cyclic AMP-dependent protein kinase is rather vexed question: while in fact a threonine residue of so called Inhibitor-1 of phosphorylase phosphatase has been found to be phosphorylated at rates comparable to other physiological substrates of the cyclic AMP-dependent kinase [22], the threonine residues replaced for suitable serine residues in clupeine Y2 [6] and in synthetic peptides [7], are quite unaffected by the kinase. Our results would indicate that threonine can actually serve as a fairly good substrate for the kinase, though poorer than serine according to the fast phosphorylation of A4S in comparison to A4. In particular the K_m values of peptide A4, including only a threonine target, are consistent with the values reported for a rather similar threonine containing heptapeptide [8] and its V is not much lower than those obtained with the serine-containing substrates (see Table III). The failure to phosphorylate some other apparently suitable threonine might be accounted for by additional structural features especially required for the phosphorylation of threonine: it should be noted for instance that a threonine residue unaffected by the cyclic AMP-dependent kinase in a pentapeptide [7] occupies the penultimate position, while the threonine residues phosphorylated in the present paper and by other authors [8] are located at the last but two position of hexa- and heptapeptides, respectively. Moreover, the threonine residue physiologically affected by the cyclic AMP-dependent kinase in the Inhibitor 1 of phosphorylase phosphatase [22] is embedded between two proline residues and included within a predicted β-turn [23], whereas the unaffected threonine residue of clupeine Y2 [6] is not included within a β-turn but rather within a predicted α-helix region [24]. The importance of the β-turn for determining the phosphorylation by the cyclic AMP-dependent kinase has been suggested [21,23]: it is possible that such a requirement might be especially mandatory in the case of threonine residues.

Finally our results provide the first evidence, as far as we are aware, of adjacent residues simultaneously phosphorylated by the cyclic AMP-dependent protein kinase, to give di-phosphorylated sites, like SerP-ThrP in peptide A1 and, probably, SerP-SerP in peptide B. It should be noted however that the ratio between di-phosphorylated and mono-phosphorylated peptides ($A1\text{-}P_2/A1\text{-}P$) does not increase during incubation, in parallel with the increase of phosphate incorporation. This is to be expected assuming that A1-P is less suitable than A1 for binding to the kinase and thus undergoing a second phosphorylation. The $A1\text{-}P_2/A1\text{-}P$ ratio however can be drastically enhanced either by increasing ATP or by decreasing the concentration of A1 (see Table IV). These data, while indicating that the formation of diphosphorylated peptides is largely dependent on experimental conditions, are also consistent with the hypothesis that $A1\text{-}P_2$ originates from A1 through a single catalytic event ($A1 + 2\ ATP \rightarrow A1\text{-}P_2 + 2\ ADP$) rather than from free monophosphorylated A1-P as the final result of a two-step mechanism. Anyway, since phosphorylated clusters, like $(Ser\text{-}P)_2$ and $(Ser\text{-}P)_3$, have been found in native protamines [12], it is theoretically possible that the cyclic AMP-dependent kinase might be involved in vivo in such processes. It should be underlined however that, as pointed out by Shenolikar and Cohen [6] also protamine kinases independent of cyclic AMP are likely to be involved in the physiological phosphorylation of such molecules. This may account for the failure of peptide C to serve as a substrate of the cyclic AMP-dependent kinase, whereas the similar sequence Arg-Arg-Val-Ser-$(Arg)_5$, also lacking any C terminal hydrophobic residue, is found phosphorylated in the trout testis iridines [12].

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