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Ordered phosphorylation of a duplicated minimal recognition motif for cAMP-dependent protein kinase present in cardiac troponin I

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Cardiac troponin I contains two adjacent serines in sequence after three arginine residues thus making up a minimally duplicated recognition motif for cAMP-dependent protein kinase. In a synthetic peptide, PVRRRSSANY, the two serine residues are phosphorylated sequentially with the intermediate formation of a monophosphorylated species according to the following reaction sequence:

Peptide $\xrightarrow{k_1}$ Peptide–P $\xrightarrow{k_2}$ Peptide–P_2. The calculated rate constants are: $k_1 = 0.435 \cdot min^{-1}$ and $k_2 = 0.034 \cdot min^{-1}$. Sequence analyses of the monophosphopeptide and its tryptic fragments show that the predominant monophosphoform carries phosphate at the second serine.

Cardiac troponin I; Troponin I peptide; cAMP-dependent protein kinase; Phosphoserine; Ordered phosphorylation; Tryptic cleavage

1. INTRODUCTION

Phosphorylation of cardiac troponin I occurs upon β-adrenergic stimulation [1]. In rat heart increase in force development correlates with incorporation of approximately 1 mol of phosphate per mol of troponin I [1]. The N-terminal region of cardiac troponin I, however, contains two serine residues both of which can be phosphorylated [2]. These two adjacent serine residues are preceded by three arginine residues in four species, bovine, rabbit, rat and man [3–6]; a downstream alanine is absent in human cardiac troponin I [4]. From bovine, rabbit and human heart, troponin I has been isolated in non-, mono- and bisphosphorylated forms [2,4]. In the test tube, cAMP-, cGMP-dependent protein kinase, as well as protein kinase C, phosphorylate both serine residues without an apparent specificity towards one or other of these two hydroxy amino acids [2]. The product of troponin I phosphorylation in vitro is the bisphosphorylated form. To date it is not known how monophosphorylated cardiac troponin I is generated.

In this report we have employed the synthetic peptide, PVRRRSSANY, derived from the phosphorylation domain of cardiac troponin I. It will be shown that cAMP-dependent protein kinase predominantly phosphorylates the second serine residue before touching the first one.

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2. EXPERIMENTAL

A solid-phase Cambridge Research Biochemical Pepsynthesizer II was employed containing 1 g of *t*-butyl-Tyr-pepsyn KA (Milli Gen, Eschborn, Germany) in the column. For coupling, a 4-fold excess of the appropriate Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acid (pentafluorophenylester, in case of serine oxo-benzo-triazolester) dissolved in dimethylformamide was applied. The side chain of arginine was protected by a 4-methoxy-2,3,6-trimethylphenylsulfonyl group. Serine carried a *t*-butyl group. For de-protection of the N- terminal Fmoc group, the resin was washed with 20% piperidine in dimethylformamide. Following synthesis the peptide resin was washed with dimethylformamide, *n*-amylalcohol and diethylether. After drying and 10 min pre-incubation with the scavengers, thioanisol and ethanedithiol (2:1, by volume), de-protection and cleavage of the peptide from the resin were carried out with trifluoroacetic acid and trifluoromethanesulfonic acid (10:1, by volume). The peptide was precipitated in pre-cooled *t*-butyl-methylether. After centrifugation, the precipitate was dried in vacuo. The solubilized peptide was purified preparatively by HPLC on reverse-phase C18 material. A linear gradient was used consisting of 0.09% trifluoroacetic acid (solvent A), and 0.08% trifluoroacetic acid and 84% acetonitrile (solvent B). Purity and quantity of the peptide were determined by amino acid and sequence analysis.

Troponin was isolated from bovine heart according to the method of [7] modified by [8]. Separation of the troponin I subunit was carried out as described previously [9]. The phosphate content of holotroponin and troponin I was determined by the method of [10].

Phosphorylation of troponin I, the troponin complex or the peptide (12–15 μM) by cAMP-dependent protein kinase (100–250 U/ml, catalytic subunit, Sigma) was carried out at 30°C in 50 mM morpholinopropane sulfonic acid, pH 7.0, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP, 1 mM DTE and, in the case of the holocomplex, additionally 100 mM KCl. Incorporation of [^{32}P]phosphate was determined by the method of [11].

The synthetic peptide and its phosphorylated forms were digested with trypsin as described [9]. The generated fragments were separated on a C18 218 TP 5415 Vydac reverse-phase HPLC column (4.6×150 mm). A linear gradient from 5 to 30% solvent B in solvent A over 25 min was employed at a flow rate of 1 ml/min. The absorbance was recorded at 214 nm.

Prior to amino acid analysis phosphopeptides were prepared for *S*-ethylcysteine analysis as described previously [9,12]. Amino acid analysis was performed as described [13]. Edman degradation of the synthetic peptide and its phosphorylated forms was carried out as described [2]. To localize the [^{32}P]phosphate in the amino acid sequence of a phosphopeptide, the method of [14] was employed with a modification as described by [2].

3. RESULTS

Phosphorylation of a synthetic peptide, PVRRRSSANY, corresponding to the phosphorylation domain of bovine, rabbit and rat cardiac troponin I by the catalytic subunit of cAMP-dependent protein kinase is compared to that of isolated troponin I and holotroponin. 1.7 mol of phosphate/mol of synthetic peptide are incorporated within 30 min, but further phosphorylation is very slow (Fig. 1). A somewhat slower time–course is observed when isolated cardiac troponin I is employed; in contrast, phosphorylation of troponin I present in the holocomplex occurs more rapidly.

Reverse-phase HPLC allows the separation of the mixture of products generated during phosphorylation of the peptide for 3 or 90 min (Fig. 2). The substrate, the non-phosphorylated peptide (I), elutes at 9.5% solvent B. During phosphorylation, the amount of non-phosphorylated peptide decreases and finally disappears completely. Two new peptides (II and III) appear, eluting successively at 1 and 2% lower concentrations of solvent B. The amino acid composition of peptide I and that of the two phosphopeptides II and III is shown in Table I. As expected, peptide I, the substrate, contains no phosphoserine, whereas the amount of phosphoserine in peptide II extrapolates to 1 mol of phosphoserine/mol of peptide and that of peptide III to 2 mol of phosphoserine/mol of peptide (Table I).

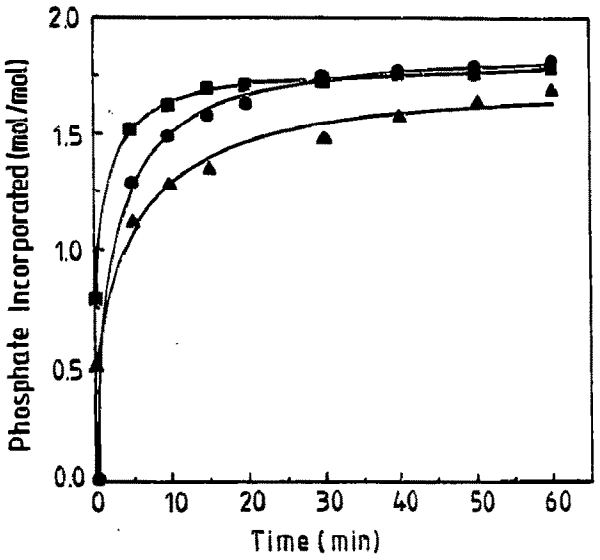

Fig. 1. Phosphorylation of the synthetic peptide PVRRRSSANY, the cardiac holotroponin complex and the cardiac troponin I subunit by cAMP-dependent protein kinase. Phosphorylation of each substrate (15 μM) by the catalytic subunit of cAMP-dependent protein kinase (250 U/ml) was carried out as described in section 2. The endogenous phosphate content of the holocomplex (■) before incubation with protein kinase was 0.8 mol of phosphate/mol of troponin I. The employed troponin I subunit (▲) contained 0.5 mol of phosphate/mol of protein. The synthetic decapeptide (●) was free of endogenous phosphate.

Employing this separation technique the time–course of appearance and disappearance of all species formed during phosphorylation can be followed. The amount of each species was determined either by measuring the [^{32}P]phosphate content or by analyzing the amino acid composition. The non-phosphorylated substrate disappears almost completely within 10 min; the amount of monophosphorylated peptide rises quickly and disappears more slowly (Fig. 3). The monophosphorylated form reaches maximally ca. 80% of the total amount of peptide employed. The bisphosphopeptide appears after a short lag phase; its increase correlates with the decrease of the monophosphorylated form. This observation suggests an ordered phosphorylation occurring first at one serine and consecutively at the second serine residue. Therefore, the following reaction mechanism can be postulated:

$$\text{Peptide} \xrightarrow[\text{ATP}\ \text{ADP}]{k_1} \text{Peptide–P} \xrightarrow[\text{ATP}\ \text{ADP}]{k_2} \text{Peptide–P}_2$$

Peptide–P and Peptide–P_2 represent the mono- and bisphosphorylated peptides, respectively. Assuming that ATP remains constant during phosphorylation, these reactions can be described by the following set of differential rate equations:

$$d[\text{Peptide–P}]/dt = [\text{Peptide}]\cdot k_1 - [\text{Peptide–P}]\cdot k_2$$
$$d[\text{Peptide–P}_2]/dt = [\text{Peptide–P}]\cdot k_2$$
$$d[\text{Peptide}]/dt = -d[\text{Peptide-P}]/dt - d[\text{Peptide–P}_2]/dt$$

Table I

Amino acid analysis of different phosphorylated peptides before and after tryptic digestion

Amino acid	Peptide							
	I	Ia	Ib	II	IIa	IIb	III	IIIa
Asx	0.8	1.0	1.0	0.9	0.9	1.0	0.8	0.8
Ser	1.8	1.8	1.9	0.9	0.9	0.9	–	–
Ser(P)	–	–	–	0.9	0.8	0.8	1.7	1.5
Arg	2.7	–	1.1	2.6	1.1	2.1	2.7	1.9
Ala	1.1	0.9	1.0	1.0	0.9	0.9	1.0	0.9
Pro	1.3	–	–	1.1	–	–	1.2	–
Tyr	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Val	1.1	–	–	0.9	–	–	1.0	–

Amino acid analyses before and after tryptic digestion following conversion of phosphoserine to *S*-ethylcysteine were carried out in duplicate as described in section 2. Peptide Ia, Ib, IIa, IIb and IIIa were the serine-containing fragments after tryptic digestion of the corresponding peptide I, II or III. Values were not corrected for destruction and slow liberation.

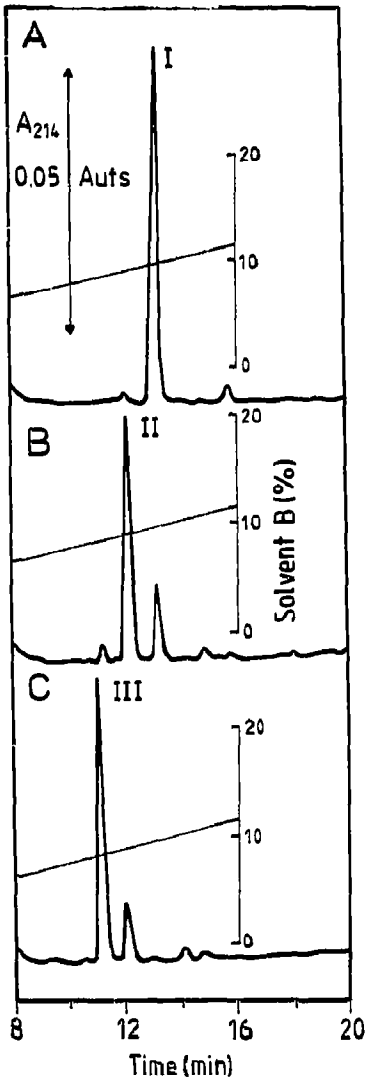

Fig. 2. HPLC analyses of the synthetic peptide phosphorylated by cAMP-dependent protein kinase. The peptide PVRRRSSANY (12 μM) was phosphorylated with cAMP-dependent protein kinase (100 U/ml) as described above (Fig. 1). After 0 min (A), 3 min (B) and 90 min (C) incubation, aliquots containing 1.6 nmol of peptide were separated on a reverse-phase HPLC column as described in section 2.

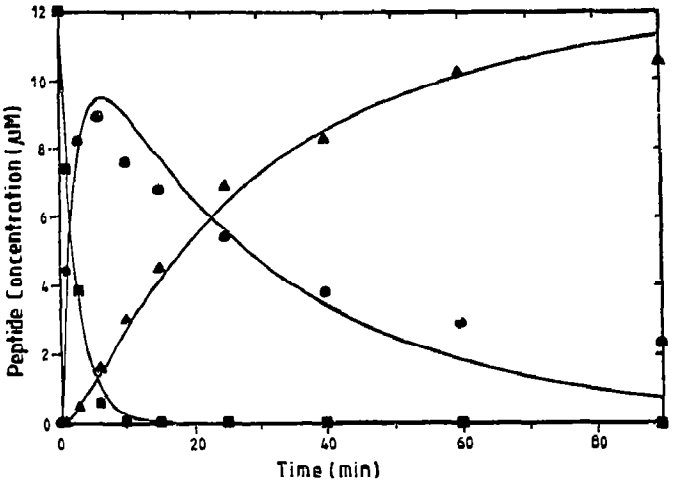

Fig. 3. Phosphopeptide formation during phosphorylation of the synthetic peptide PVRRRSSANY. Phosphorylation conditions and isolation of the phosphopeptides are described above (Fig. 2). The amount of each peptide, the non-phosphorylated substrate (■), the monophosphorylated (●), and the bisphosphorylated form (▲), is determined by [^{32}P]phosphate content or by amino acid analysis as described in section 2. The curves calculated with the rate constants, k_1 and k_2 (given in the text), represent the optimal adaptation to the experimental data employing the software MATLAB.

The system was solved by using the numerical software MATLAB. The optimal adaptation to the experimental data was achieved employing the rate constants $k_1 = 0.435\ \text{min}^{-1}$ and $k_2 = 0.034\ \text{min}^{-1}$ (Fig. 3).

In this reaction sequence potentially two monophosphorylated species can be formed, namely the one phosphorylated on the first or on the second serine residue. Therefore, the monophosphorylated peptide is isolated by HPLC as shown in Fig. 2. During Edman degradation phosphoserine forms dehydroalanine and inorganic phosphate thus allowing the determination of the step at which [^{32}P]phosphate is liberated. The autoradiogram following high-voltage iontophoresis shows that a low amount of inorganic phosphate is liberated from the first serine (Fig. 4, peptide II). At the position of the second serine more than twice the amount of [^{32}P]phosphate is found. This indicates that a minor amount of phosphate is incorporated into the first serine, and the major amount into the second serine. Apparently, these two monophosphorylated species are not separated by HPLC, which prevents an easy quantification of each monophospho form.

As an alternate approach, these peptides were digested with trypsin. The bisphosphorylated peptide is cleaved exclusively after the first arginine yielding the fragment IIIa, RRS(P)S(P)ANY (Fig. 5C and D, Table I). In contrast, the non-phosphorylated peptide yields SSANY as the major product fragment Ia, resulting from cleavage after the third arginine. Only a minor part of this peptide is cleaved between the second and the third arginine residue, which produces fragment Ib (Fig. 5A and D, Table I). Digestion of the monophosphopeptide II, which represents a mixture of both possible monophospho species, results in two major fragments IIa and IIb; IIa contains one arginine residue and IIb contains two (Fig. 5B and D, Table I). Peptide IIa is homogeneous; it does not liberate inorganic phosphate during Edman degradation of the first serine residue; radioactivity is liberated, however, during the subsequent cycle, i.e. upon degradation of the second serine residue (Fig. 4). During Edman degradation Peak IIb behaves like the non-digested monophosphorylated peptide II; [^{32}P]phosphate is liberated mainly upon degradation of the second serine residue. Thus, trypsin cleaves the monophosphopeptide- containing phosphoserine in the second position after the first or

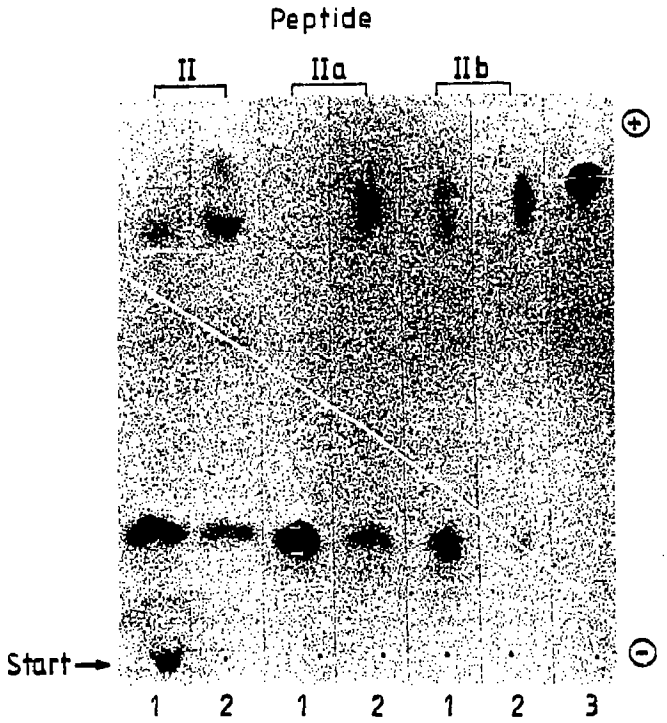

Fig. 4. Localization of [^{32}P]phosphoserine in the amino acid sequence of the synthetic monophosphopeptide and its tryptic fragments. The isolated [^{32}P]monophosphopeptide II (see Fig. 2) and the tryptic fragments IIa and IIb (see Fig. 5) are subjected to Edman degradation. Sequence analysis is interrupted after the first and the second serine, respectively. Liberated [^{32}P]phosphate is identified as described by [2]. The autoradiogram shows the position of the liberated [^{32}P]phosphate and the radioactively labelled residual peptide. Lane 1, radioactivity distribution after interruption at the first serine, lane 2, at the second serine residue, and lane 3 is the [^{32}P]phosphate standard.

the second arginine residue (see Fig. 5D), whereas it cleaves the monophosphopeptide-carrying phosphoserine at the first position exclusively after the first arginine. Again, the two resulting fragments composed of two arginine residues and one phosphoserine, irrespective of the position (peak IIb), are not separated by reverse-phase HPLC chromatography.

4. DISCUSSION

The minimal recognition motif for cAMP-dependent protein kinase is generally accepted to be RRXS [15]. Cardiac troponin I contains this motif in a duplicated form represented by the sequence RRRSS. The three basic amino acids are arranged in such a way that the first, as well as the second, serine residue fulfills the requirement of the minimal recognition motif (see above). As demonstrated here, both serine residues are phosphorylated by cAMP-dependent protein kinase when a synthetic peptide comprising the duplicated minimal recognition motif is employed. In comparison with other substrates this duplication does not drastically change the properties as substrate: the K_M value of 4 μM determined by measuring the initial [^{32}P]phosphate incorporation rate (not shown) is similar to that of 8 μM reported for another synthetic peptide, kemptide [16].

There are principally two possibilities for a duplicated substrate motif to be positioned in the catalytic center of the protein kinase: either the first or the second serine is phosphorylated (for description of the catalytic center compare [17,18]). The results shown here suggest that the preferred position is the one in which the second serine can accept the phosphate group. When analyzing the mixture of monophosphorylated peptides it can clearly be seen that the major part of the incorporated [^{32}P]phosphate is found at the second serine and only a minor part at the first serine (see Fig. 4). The cleavage pattern of these monophosphopeptides with trypsin is in agreement with this conclusion (see Fig. 5). This protease is unable to cleave a peptide bond after arginine or lysine if a phosphoserine is located two positions downstream (compare Fig. 5, peptides IIa, IIb and IIIa). This pattern was also observed by Parker et al. [19] and Picton et al. [20]. The situation is somewhat more complicated here due to the presence of multiple arginine residues; they lead to additional cleavage products by clipping further upstream from phosphoserine. Nevertheless this analysis also leads to the conclusion that the second serine is phosphorylated first.

Meanwhile, accumulation of a monophosphoform during phosphorylation of troponin I present in the holocomplex by cAMP-dependent protein kinase has been detected (D. Soprani, personal communication). This suggests that such an ordered sequential phosphorylation of troponin I also occurs in the native protein. The opposite behavior has been observed by phosphorylating skeletal muscle troponin T with phosphorylase kinase [21]. In analogy to cardiac troponin I two serine residues, located in position 149/150 and 156/157, are phosphorylated: a monophosphoform has never been detected during phosphorylation. In this instance, however, a monophosphoform is formed upon de-phosphorylation by a protein phosphatase. Thus, differential phosphorylation or de-phosphorylation can generate intermediate monophosphorylated species which may represent important differences in regulation by protein kinase or protein phosphatase.

As a consequence of sequential phosphorylation or de-phosphorylation multiple forms are produced. The number of forms, f, increases according to $f = 2^n$ where n is the number of phosphorylatable residues, in this case serine. In such a system each species may have a different function. Alternatively, only two functional states may exist which are represented by the fully dephosphorylated and the fully phosphorylated proteins. Partially phosphorylated forms then could be intermediates in this functional transition, exhibiting the same function to different degrees. Cardiac troponin I might be the optimal system to analyze such a situation since

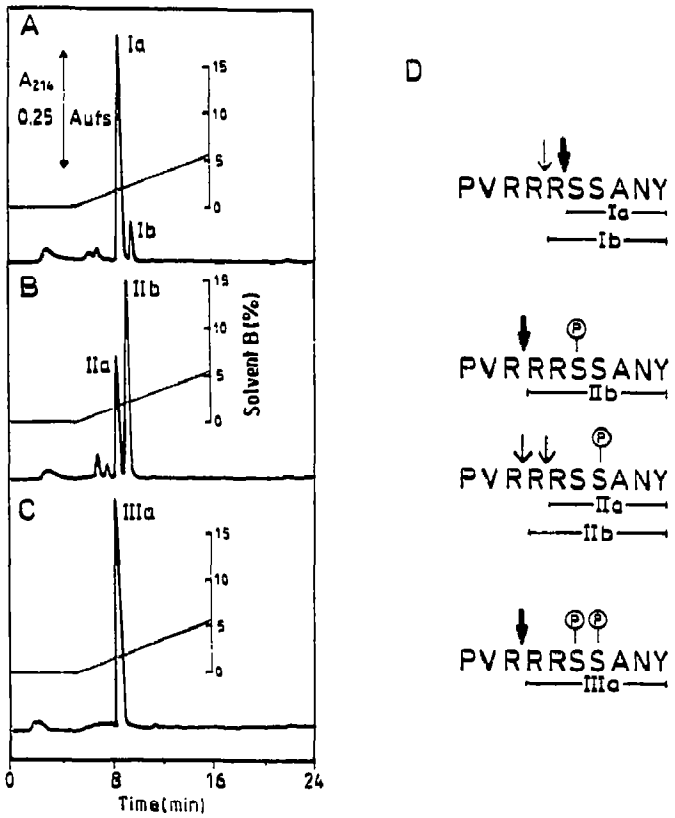

Fig. 5. Separation of tryptic fragments obtained from non-, mono- and bisphosphorylated peptides and the resulting cleavage sites of trypsin. 20 nmol of peptide I (A), 33 nmol of peptide II (B) and 20 nmol of peptide III (C) were digested with trypsin as described by [9]. The fragments were separated on a reverse-phase HPLC column as described under section 2. The cleavage sites of trypsin in the synthetic decapeptide and its phosphoforms (D) are shown (see Fig. 4, Table I). The major position at which the peptide was cleaved is marked by a thick arrow, and the minor cleavage site by a thin arrow.

only four states have to be analyzed due to the presence of two phosphoserine residues.

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