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A common motif of two adjacent phosphoserines in bovine, rabbit and human cardiac troponin I

Karin Mittmann, Kornelia Jaquet and Ludwig M.G. Heilmeyer Jr

Institut für Physiologische Chemie, Abteilung für Biochemie Supramolekularer Systeme, Ruhr-Universität Bochum, Postfach 102148, D-4630 Bochum, FRG

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From rabbit and human cardiac troponin I N-terminal mono and bisphosphorylated peptides were isolated which were obtained from Lys-C proteinase digests. Two adjacent phosphoserine residues could be localized in each phosphopeptide following further tryptic digestion. The previously published sequence of rabbit cardiac troponin I had to be corrected. Two adjacent phosphoserine residues are a common motif in the very similar sequences of bovine, rabbit and human cardiac troponin I. The N-terminal sequences are: AcADRSGGSTAG DTVPAPPPVR RRS*(P)*S*(P)*ANYRAY ATEPHAK (rabbit), AcADESTDA-AG EARPAPAPVR RRS*(P)*S*(P)*ANYRAY ATEPHAK (bovine), (Ac,A,D/N,G,S,S,D/N,A,A,R) EPRPAPAPIR RRS*(P)*S*(P)*-NYRAY ATEPHAK (human).

Cardiac troponin I; Phosphorylation site; Phosphoserine; N-terminal sequence; Rabbit heart; Human heart

1. INTRODUCTION

Upon β-adrenergic stimulation cardiac troponin I, the inhibitory subunit of troponin, is phosphorylated in mammalian hearts [1,2] which is believed to occur on rabbit troponin I at serine-20 [3]. Therefore, a correlation between phosphorylation of this single amino acid and change in contractility was expected which, however, could not be clearly demonstrated in hormonally stimulated perfused hearts [4].

Freshly isolated troponin I from both rabbit and bovine heart contains 1.5–1.9 mol of phosphate per mol of protein [5,6]. Therefore, it was concluded that in addition to phosphoserine-20 a second phosphate residue of unknown location is bound in the N-terminal sequence stretching from amino acid 1 to 48 [3]. Recently, two adjacent phosphoserine residues in position 23 and 24 were identified in troponin I from bovine heart [6]. However, this phosphorylation domain differs considerably in its sequence from the phosphorylation site of rabbit troponin I [7,8]. The amino acid arrangement near phosphorylation sites form recognition domains for protein kinases which enhance the pressure to conserve such sites during evolution. The presence of two adjacent phosphoserine residues in bovine and only one phosphoserine residue in rabbit troponin I contradicts this hypothesis.

Correspondence address: L.M.G. Heilmeyer Jr, Institut für Physiologische Chemie, Abteilung für Biochemie Supramolekularer Systeme, Ruhr-Universität Bochum, Postfach 102148, D-4630 Bochum, FRG

Skeletal muscle troponin I does not contain the N-terminal region present in the heart isoform. Therefore, cardiac troponin I is an excellent heart-specific cell marker which can be detected in blood plasma of patients with acute myocardial infarction [9]. Antibodies against phosho- and dephospho troponin I may even allow to analyze the hormonal status of a heart. A prerequisite is the localization of the phosphorylation domain in human troponin I.

In the present publication the location of the second in vivo phosphorylation site of rabbit cardiac troponin I will be reported requiring a correction of the amino acid sequence. Additionally, the phosphorylation domain of human cardiac troponin I will be shown.

2. MATERIALS AND METHODS

Freshly excised hearts from rabbits, immediately frozen with liquid nitrogen, were stored at −50°C. Myocardial tissue probes from explantated human hearts were obtained from the Herzzentrum Nordrhein Westfalen, Bad Oeynhausen. Troponin I was purified from both human and rabbit heart according to [10]. About 350 μg troponin I, electrophoretically pure, were obtained from one gram wet heart. The protein-containing fractions were applied on PD-10 columns (Pharmacia) to remove urea. Troponin I fragments were isolated after endoproteinase Lys-C and trypsin digestion as described previously [6]. Phosphoserine-containing peptides were analyzed following *S*-ethylcysteine modification [6,11,12].

Protein-bound phosphate was determined by the method of [13]. Amino acid and sequence analyses were carried out as described previously [6,14]. Mass-spectra were recorded on a plasma desorption mass spectrometer in the Biomedical Center of the University of Uppsala. 1 nmol of the phosphopeptide was solubilized in 30 μl of 0.1% (v/v) trifluoroacetic acid. 7 μl of the protein solution were incubated with ethanol for 15 min before the probe was applied to the mass-spectrometer.

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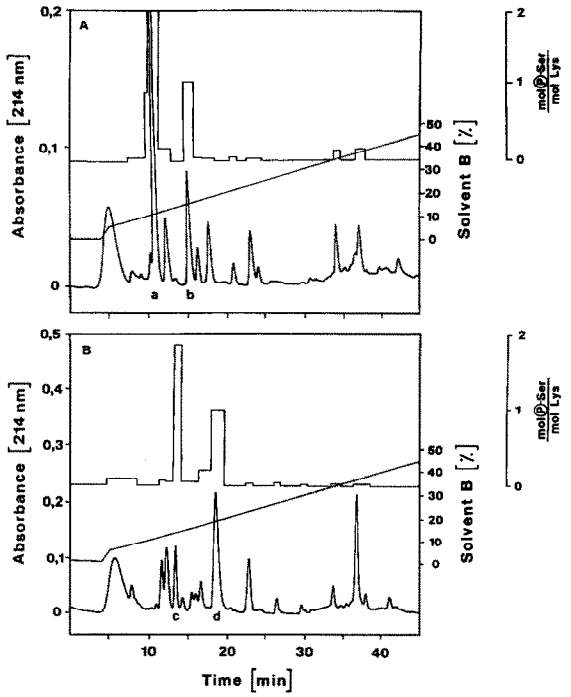

Fig. 1. Separation of endoproteinase Lys-C peptides from rabbit and human cardiac troponin I. 10 nmol of each troponin I from rabbit (A) and human cardiac muscle (B) were digested with endoproteinase Lys-C as described previously [6]. The peptide mixtures (900 μl) were separated on a pH-stable Vydac C8 column (4.6 × 250 mm) at a flow rate of 1 ml/min. A gradient was applied as indicated. Solvent A contained 0.2% hexafluoroacetone/NH_3, pH 7.8, and solvent B 84% acetonitrile, 0.03% hexafluoroacetone/NH_3, pH 7.8.

3. RESULTS

Troponin I freshly isolated from frozen rabbit heart muscle ($n = 3$) contained about 1.9 mol phosphate per mol protein whereas that isolated from frozen human heart ($n = 5$) only 0.2–1.0. In both materials phosphate was present as phosphoserine exclusively; the amount of phosphate as determined by ashing and of phosphoserine as determined by *S*-ethylcysteine, were identical within the standard error of both methods (data not shown).

To localize these phosphoserine residues in rabbit and human troponin I fragments obtained by digestion with lysine-specific proteinase were separated by reversed-phase high-performance liquid chromatography (Fig. 1). In each chromatogram two peaks, (*a* and *b* for rabbit or *c* and *d* for human), were found to contain one or two mols of phosphoserine per mol of lysine (Table I) indicating that peptides *a* and *c* represent the bisphosphorylated and peptides *b* and *d* the monophosphorylated N-termini of each troponin I, respectively. Peptides *a* and *b* from rabbit cardiac troponin I differ in mass by one phosphate group (Fig. 2). The determined molecular masses of both these peptides (4018 and 4098) agree with the amino acid composition (Table I). The calculated molecular mass of the N-terminal Lys-C peptide (3444) according to the published rabbit cardiac troponin I sequence [7] is 574 Da smaller than the determined mass of peptide *b* (4018). Therefore the amino acid composition as well as

Table I

Amino acid compositions of phosphoserine-containing peptides from troponin I

Amino acid	Rabbit cardiac					Human cardiac			
	Peptide			N-terminus		Peptide		Peptide	
	a	*aI*	*b*	1–36	1–31	*c*	*cI*	*d*	*dI*
Asx	1.8	0.9	1.8	3	3	1.6	0.7	1.2	0.7
Glx	2.3	–	2.4	3	3	1.7	–	1.2	–
Ser	1.0	–	2.2	1	1	1.7	–	2.5	0.8
Ser(P)	2.0	2.1	1.1	2	1	2.1	2.0	1.0	1.2
Gly	1.2	–	1.3	1	1	1.2	–	1.4	–
His	1.1	–	0.9	1	1	1.0	–	1.0	–
Arg	6.4	3.3	5.8	6	5	5.7	3.1	5.9	1.9
Thr	(2.1)	–	(2.0)	1	1	(1.5)	–	(1.4)	–
Ala	9.7	1.3	9.6	10	9	7.4	–	7.6	–
Pro	4.0	–	4.1	4	3	4.8	–	5.1	–
Tyr	2.2	1.0	1.8	2	1	2.0	1.0	2.1	1.0
Val	1.3	–	1.2	1	1	–	–	–	–
Ile	–	–	–	–	–	0.9	–	1.0	–
Lys	1.0	–	1.0	1	1	1.0	–	1.0	–
Mass	4098	n.d.	4018	4098	3444	n.d.	n.d.	n.d.	n.d.

Amino acid analyses following conversion of phosphoserine to *S*-ethylcysteine were carried out in duplicate. The amountt of amino acids of the Lys-C peptides *a–d* was related to 1 mol lysine and of the tryptic peptides *aI*, *cI* and *dI* to 1 mol tyrosine. Values were not corrected for destruction and slow liberation. The determined amount of threonine is too high, caused by an unknown contamination in all samples and therefore were disregarded. The amino acid composition of the N-terminus 1–31 from rabbit cardiac troponin I containing phosphoserine-20 [3] was taken from [7]. The calculated and determined masses of the N-terminus are given (compare Fig. 2). n.d. = not determined.

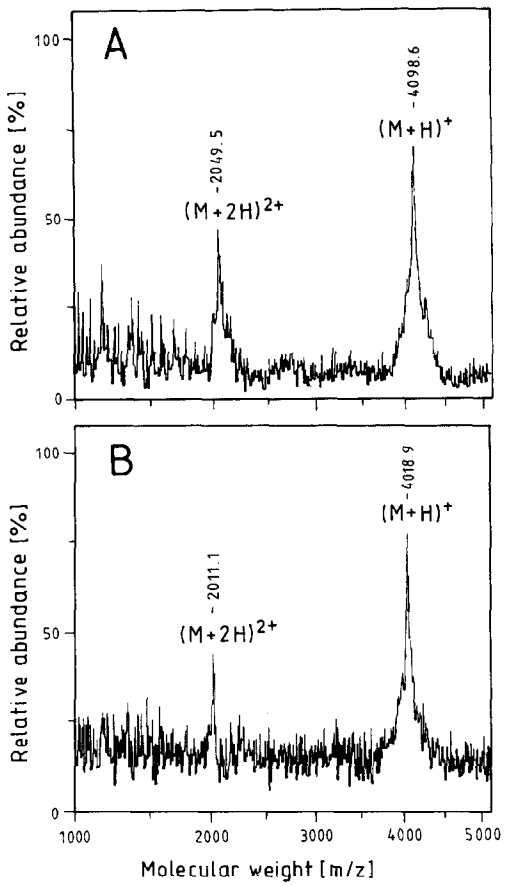

Fig. 2. Plasma desorption mass spectra of the phosphorylated N-terminal peptides *a* and *b* from rabbit cardiac troponin I. 230 pmol of each peptide *a* (A) and peptide *b* (B) were applied. The calculated masses of 4098 and 4018, respectively, according to amino acid composition are given in Table I.

the molecular mass indicate that approximately 5 amino acids are present additionally in this peptide.

The isolated N-terminal Lys-C peptides *a*, *c* and *d* were further digested with trypsin. The yield of peptide *b* was too small for further digestion since 90% of troponin I from rabbit was obtained in the bisphosphorylated form. Peptide *aI* from rabbit (Fig. 3A), as well as peptide *cI* from human cardiac troponin I (Fig. 3B) showed a characteristic ratio of approximately 3 mol arginine to 2 mol phosphoserine (Table I). The monophosphorylated peptide *dI* (Fig. 3C) derived from human cardiac troponin I contained only two arginine residues. Characteristically the human peptides *cI* and *dI* contained no alanine (Table I). Following *S*-ethylcysteine modification all three tryptic phosphopeptides *aI*, *cI* and *dI* were subjected to Edman degradation. During degradation of both bisphosphorylated peptides from rabbit and human cardiac troponin I (Fig. 4A and B) nearly exclusively the phenylhydantoin derivatives of *S*-ethylcysteine (*S*-Et-Cys) were found in cycles 3 and 4. The amount of dithiothreitol-serine (DTT-Ser) was less than 20%, free serine was not detectable. Thus, in both the rabbit and the human peptides phosphoserine residues were present at these two positions. In agreement with the amino acid composition the phosphopeptide from human cardiac troponin I showed no alanine residue following the second phosphoserine residue (Fig. 4). During Edman degradation of the monophosphorylated peptide *dI* serine was found in cycle 2 and *S*-ethylcysteine in cycle 3 (Fig. 3C).

The sequence of the bisphosphorylated peptide derived from rabbit cardiac troponin I is incompatible with the published sequence [7]. However, it is identical to the sequence of the corresponding peptide from bovine cardiac troponin I. Of the five postulated additional amino acids four were identified within the tryptic phosphopeptide from rabbit cardiac troponin I (Table I). The fifth amino acid, a proline residue, was determined following alanine-16 in the peptide *aII* (Fig. 3A, sequence data not shown). The corresponding tryptic fragment from human cardiac troponin I showed a

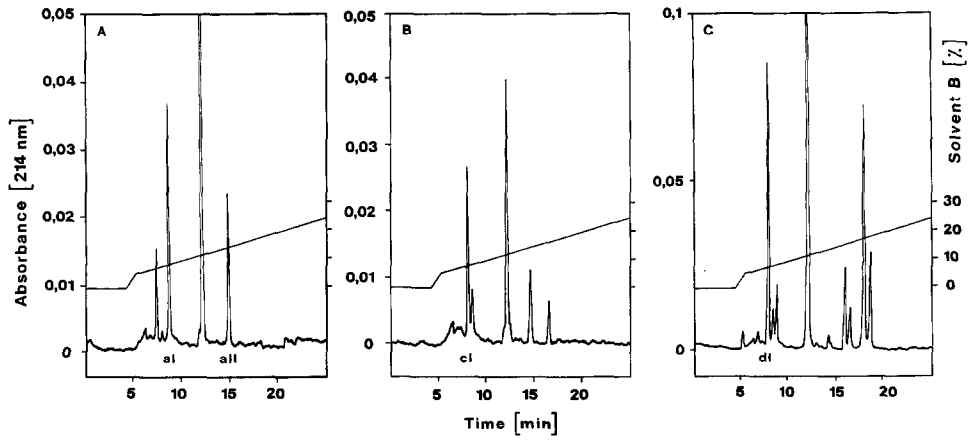

Fig. 3. Separation of the tryptic fragments from the phosphoserine-containing troponin I peptides *a* (rabbit), peptide *c* and *d* (human). 1.0 nmol peptide *a* (A), 0.9 nmol peptide *c* (B) and 2.3 nmol peptide *d* (C) were digested with trypsin as described previously [6]. The digestion mixtures were injected on a Vydac C18 column 218TP5415 (4.6 × 150 mm) using the indicated gradient from solvent A (0.09% trifluoroacetic acid) to solvent B (84% acetonitrile, 0.08% trifluoroacetic acid). The flow rate was 1 ml/min.

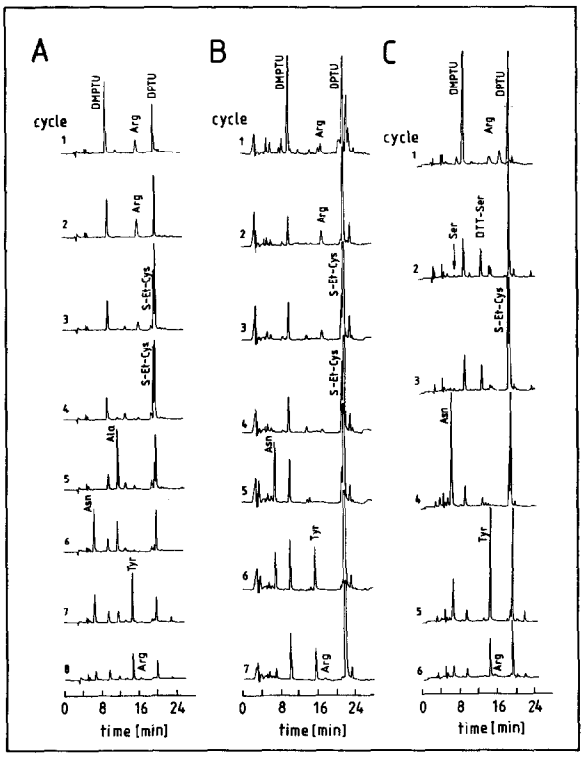

Fig. 4. Sequence analysis of the tryptic peptides *aI*, *cI* and *dI*. Samples were sequenced following derivatization of phosphoserine residues to *S*-ethylcysteine. 1.2 nmol peptide *aI* (A) was applied, the repetitive yield was 68%. 0.2 nmol peptide *cI* (B) was sequenced with a resulting repetitive yield of 97%. 0.6 nmol peptide *dI* (C) was applied, the calculated repetitive yield was 84%. DMPTU, dimethylphenylthiourea; DPTU, diphenylthiourea; DTT-Ser, dithiothreitol-serine; S-Et-Cys, *S*-ethylcysteine.

very similar amino acid sequence: alanine-11 is exchanged against proline and valine-18 against isoleucine. Sequences of N-terminal blocked tryptic peptides have not been determined.

4. DISCUSSION

Strong alkaline conditions as employed in the spinning cup sequencer results in dehydroalanine formation by β-elimination of phosphoserine. When present N-terminally the formed dehydroalanine rearranges and yields a pyruvyl end group which blocks further sequencing. It explains why extreme difficulties existed in determining the location of phosphoserine residues in the primary structure. The *S*-ethylcysteine method overcomes this problem. As demonstrated in Fig. 4 two adjacent phosphoserine residues or a serine residue followed by phosphoserine were easily detected in the rabbit and human troponin I sequence.

Indeed, due to the correction of the rabbit troponin I sequence all three sequences, that of bovine, rabbit and human, are very similar (Fig. 5). Characteristically, there exists a proline-rich region upstream of the phosphorylation domain formed by three arginine and two serine residues. The cluster of helix breaking proline residues may change the structure of the N-terminus in such a way that the following phosphorylation domain can adapt to the catalytic center of protein kinases. Indeed, these two serine residues can be phosphorylated by the cAMP-dependent, cGMP-dependent protein kinase and protein kinase C [15]. Furthermore, the bound phosphate groups are exposed on the surface of the protein as can be judged from ^{31}P NMR studies [16]. In vitro phosphorylation of troponin I yields the bisphosphorylated product exclusively [15] vice versa dephosphorylation yields the completely dephosphorylated material. The monophosphorylated species are not found (Jaquet and Heilmeyer, unpublished). In contrast, both monophosphorylated forms of troponin I are found in the freshly isolated protein [6,15]. It indicates that these two serine residues are phosphorylated and dephosphorylated differential-

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                                                    23 24          37
                                                     \ /
Bovine cardiac [6,8]          AcADRSGGSTAG DTVPAPPPVR RRSSANYRAY ATEPHAK
                                                      PP

                                                    20             31
Rabbit cardiac [3,7]          AcADESRDA-AG EARPAPA-VR R-S--D-RAY ATEPHAK
                                                      P

                                                    22 23          36
                                                     \ /
Rabbit cardiac, corrected     AcADESRDA-AG EARPAPAPVR RRSSANYRAY ATEPHAK
                                                      PP

                                                    22 23          36
                                                     \ /
Human cardiac  (Ac,A,D/N,G,S,S,D/N,A,A,R) EPRPAPAPIR RRSS-NYRAY ATEPHAK
                                                      PP
```

Fig. 5. Comparison of the N-terminal phosphorylation sites from rabbit, bovine and human cardiac troponin I. The amino acid sequences were aligned to maximize homology. No sequence could be determined for the N-terminal blocked peptide, therefore the amino acid composition is given in parentheses. The N-terminal acetylgroup of rabbit troponin I was determined by plasma desorption mass spectrometry.

ly. It explains why no linear correlation between phosphate incorporation and change in contractility was observed in perfused rabbit hearts [4].

Heart contractility is influenced by two hormonal signals triggered by α_1- and β-adrenergic receptor occupation. The corresponding two signal pathways merge at troponin; maybe, differential phosphorylation of two serine residues on troponin I is the molecular equivalent of signal attenuation observed in heart perfusion studies [17].

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