

# Characterization of the Cardiac Troponin I Phosphorylation Domain by $^{31}\text{P}$ Nuclear Magnetic Resonance Spectroscopy<sup>†,‡</sup>

Kornelia Jaquet,<sup>§</sup> Katrin Korte,<sup>||</sup> Klaus Schnackerz,<sup>⊥</sup> Karel Vyska,<sup>§</sup> and Ludwig M. G. Heilmeyer, Jr.\*<sup>||</sup>

*Krankenhausbetriebsgesellschaft mbH, Herzzentrum Nordrhein-Westfalen, 32545 Bad Oeynhausen, Germany, Abteilung für Biochemie Supramolekularer Systeme, Institut für Physiologische Chemie, Ruhr-Universität Bochum, 44780 Bochum, Germany, and Theodor-Boveri-Institut für Biowissenschaften, Physiologische Chemie I, Universität Würzburg, Am Hubland, 97074 Würzburg, Germany*

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**ABSTRACT:** Cardiac holotroponin can be phosphorylated at serine 23 and/or 24 in the heart-specific region of bovine troponin I. When isolated freshly it is composed of a mixture of non-, two mono-, and bisphosphorylated species. At neutral pH the monophosphorylated form carrying phosphate at serine 24 yields a resonance signal at 4.6 ppm and that carrying phosphate at serine 23 at 4.4 ppm; the two phosphate groups of the bisphosphorylated form yield only one  $^{31}\text{P}$ -NMR signal at 4.2 ppm. From the chemical shift dependence on pH,  $\text{pK}_a$  values have been estimated to be 5.3 and 5.6 for the phosphate groups at serine 24 and serine 23, respectively. Both phosphates of the bisphosphorylated form exhibit very similar  $\text{pK}_a$  values of approximately 5.8. Separation of bisphosphotroponin I from the complex results in a downfield shift and the appearance of two  $^{31}\text{P}$ -NMR signals at positions comparable to those of the two monophospho forms. Complex formation of cardiac troponin I with C or T does not alter the spectrum obtained with isolated troponin I; however, the original troponin spectrum is restored by reconstitution of the holocomplex from all three components T, I, and C. Two signals are also observed with a bisphosphorylated synthetic peptide [PVRRRS(P)S(P)ANYR] representing the phosphorylation domain.  $\text{pK}_a$  values of about 5.3 and 5.6 have been determined for serine 7 (corresponding to serine 24 of troponin I) and serine 6 of the peptide (corresponding to serine 23 of troponin I).

Freshly isolated cardiac troponin, a regulatory protein of the thin filament, contains up to 2 mol of phosphate/mol of protein in the troponin T as well as in the troponin I subunit (Beier et al., 1988). Two adjacent phosphoserine residues have been identified in the heart-specific N-terminal region of troponin I (Swiderek et al., 1988).

The sequence of three arginine residues followed by two phosphoserine residues—RRRS(P)S(P)—was found in cardiac troponin I of several mammals (Mittmann et al., 1990). This domain thus forms a duplicated minimal recognition motif for the cAMP-dependent protein kinase (Mittmann et al., 1992). In intact heart, about 1 mol of phosphate/mol of cardiac troponin I is incorporated upon  $\beta$ -adrenergic stimulation which is correlated with an enhancement of force (England, 1976). Mechanistically, phosphorylation of troponin I may change the calcium affinity of troponin C (Holroyde et al., 1980).

Two phosphorylation sites potentially can give rise to four species: non-, two mono-, and bisphosphorylated forms. It is unknown if each species carries a distinct function, and if so, how each phosphorylation signal is transduced intramolecularly from the phosphorylation domain to the  $\text{Ca}^{2+}$ -binding domain(s) on troponin C.

$^{31}\text{P}$ -NMR spectroscopy provides an excellent tool to observe directly changes in the chemical environment of a phosphate group. In order to perform these measurements, cardiac troponin must, however, be obtained free of contaminating protein phosphatase and protease activities (Beier et al., 1988). Employing such a material, four signals are observed, one originating from troponin T and three from troponin I (Swiderek et al., 1990).

Sequence analysis demonstrates that isolated cardiac troponin I is composed of a mixture of all four forms, representing the possible phosphorylation states mentioned above (Swiderek et al., 1990). An assignment of the observed  $^{31}\text{P}$ -NMR signals to distinct phosphoforms has not yet been made. In this publication we will report which factors give rise to the three-signal spectrum of troponin I. Furthermore, it will be shown that one of the three troponin I signals is due to an interaction of troponin I with other subunit(s) of the complex. The  $^{31}\text{P}$ -NMR signals observed in different synthetic phosphopeptides representing the phosphorylation domain are compared with those of the isolated troponin I subunit. This allows conclusion concerning the chemical environment of the phosphate groups in holotroponin.

## MATERIALS AND METHODS

Troponin is isolated from bovine heart according to the method of Tsukui and Ebashi (1973) modified by Beier et al. (1988). For  $^{31}\text{P}$ -NMR measurements using troponin I, I/C, T/I, and reconstituted T/I/C complexes the subunits were isolated by DEAE-Sephadex chromatography in 6 M urea according to Greaser and Gergely (1971) and for sequence analysis by hydrophobic interaction chromatography as

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\* To whom correspondence should be addressed.

<sup>§</sup> Krankenhausbetriebsgesellschaft mbH.

<sup>||</sup> Ruhr-Universität Bochum.

<sup>⊥</sup> Universität Würzburg.

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described previously (Swiderek et al., 1988). Reconstitution of the various troponin complexes (T/I, I/C, and T/I/C) was performed by gradually removing 6 M urea, in the presence of 1.25 mM  $\text{CaCl}_2$  according to Potter (1982). Complex formation from isolated subunit was verified by gel filtration over Sephadex G75 or G100 for the holocomplex. The eluted peaks were analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli (1979). Actomyosin ATPase activity was determined as described by Greaser and Gergely (1971). Phosphate of holotroponin and of isolated troponin I is determined by the method of Stull and Buss (1977). Troponin containing phosphate exclusively on the I subunit is obtained by specifically dephosphorylating the T subunit according to Villar-Palasi and Kumon (1981). After dephosphorylation the phosphate content was 0 in troponin T and about 1.4 mol in troponin I [for description of phosphate determination in the isolated subunits following separation by HPLC see Swiderek et al. (1988)]. The isolated holotroponin complex contained 2.4 mol of phosphate/mol of protein (1.0 mol of phosphate/mol of troponin T and 1.4 mol of phosphate/mol of troponin I).

The peptides PVRRRSSANYR, PVRRRSAANYR, and PVRRRASANYR are synthesized on a solid-phase Cambridge Research Biochemical Pepsynthesizer II. Fmoc-Arg-(mtr)-Pepsyn KA (1 g; MilliGen, Eschborn, FRG) is employed as column material. For coupling, a 4-fold excess of the appropriate Fmoc [9-fluorenyl(methoxycarbonyl)] protected amino acids (pentafluorophenyl ester, in the case of serine oxobenzotriazolester) is applied. Coupling conditions, subsequent cleavage from the resin, and purification of the peptide are described by Mittmann et al. (1992). Purity and quantity of the peptide are determined by amino acid and sequence analysis.

Troponin I containing less than 1 mol of phosphate/mol of protein is digested with endoprotease Lys C (1:20 by mass; Boehringer Mannheim, Mannheim, FRG). Digestion, isolation of the N-terminal phosphopeptide and its subsequent tryptic digestion (phosphopeptide:trypsin = 1:20 by mass) are performed as described by Swiderek et al. (1988). Tryptic phosphopeptides are quantified by the *S*-ethylcysteine method as described by Meyer et al. (1991). Sequence analysis is carried out on an Applied Biosystems 470A automated gas-phase sequence analyzer (Meyer et al., 1991).

Phosphorylation of troponin (21  $\mu\text{M}$ ) or the synthetic peptide (760  $\mu\text{M}$ ) is carried out at 30 °C for 1 h in 20 mM morpholinopropanesulfonic acid, pH 7.0, 10 mM  $\text{MgCl}_2$ , 1 mM dithioerythritol (DTE), 100 mM KCl, 1 mM ATP, and catalytic subunit of cAMP-dependent protein kinase (200–250 microunits/mL, Sigma, München, FRG). Prior to NMR measurements, ATP is removed from the troponin complex by extensive dialysis against 2 mM  $\text{NH}_4\text{HCO}_3$ . For concentration the protein solution is dried *in vacuo*.

The phosphorylated peptide is purified by preparative HPLC on Nucleosil MN 700- $\mu\text{m}$   $\text{C}_{18}$  (Macherey and Nagel, Düren, FRG) at a flow rate of 5 mL/min. Solvent A contains 0.09% trifluoroacetic acid and solvent B 0.08% trifluoroacetic acid in 84% acetonitrile. A linear gradient from 5% to 40% solvent B with 1%/min is employed. Absorbance is recorded at 214 nm. The amount of peptide and of phosphate is determined by amino acid analysis using the *S*-ethylcysteine method according to Meyer et al. (1991).

Spectra are recorded on a 400-MHz Bruker W 250 or on a 300-MHz Bruker AM 300 SWB. For  $^{31}\text{P}$ -NMR measurements the protein is dissolved in  $\text{D}_2\text{O}$  containing 20 mM morpholinopropanesulfonic acid, pH 7.2, 0.5 M KCl, and 1

mM DTE (Uvasol grade, Merck, Darmstadt, FRG). The pH is not corrected for  $\text{D}_2\text{O}$  content. Holotroponin (15–30 mg), 10 mg of isolated troponin I, and 1–5 mg of synthetic peptide solved in  $\text{D}_2\text{O}$  are placed in 5-mm NMR tubes. Spectra are usually recorded with broad-band proton decoupling at room temperature. A pulse width of 12.5  $\mu\text{s}$  is used; generally, sweep width is 4065 Hz with a repetition time of 1 s. Chemical shifts are quoted relative to external 85%  $\text{H}_3\text{PO}_4$  with the positive axis indicating resonances at fields lower than the reference.  $\text{Ca}^{2+}$  saturation of cardiac troponin does not influence the  $^{31}\text{P}$ -NMR signals.  $\text{Ca}^{2+}$  has been present in usual contaminating concentrations of 1–10  $\mu\text{M}$ . Furthermore, addition of chelating agents has no effect on the chemical shift nor on the line width [compare Beier et al. (1988)]; i.e., paramagnetic ions apparently are not present in the troponin preparation.

Titration curves were fitted by nonlinear regression analysis using the program GRAPH-PAD. For the  $\text{pK}_a$  values a standard deviation of about 1% was calculated.

## RESULTS

Three  $^{31}\text{P}$ -NMR signals are observed for bovine cardiac holotroponin containing approximately 1.4 mol of phosphate/mol of protein only bound to the I subunit at serine 23 and/or 24 (Figure 1A; for preparation and characterization of troponin see Materials and Methods). The phosphate content can be enhanced exclusively in the troponin I subunit by phosphorylation of troponin with the cAMP-dependent protein kinase [see Mittmann et al. (1990)]; the fully phosphorylated material then contains approximately 1.9 mol of phosphate/mol of troponin I (not shown). The  $^{31}\text{P}$ -NMR spectrum of this bisphospho form shows only one major signal at about 4.2 ppm (Figure 1B). It is recorded at a pH identical to that shown in Figure 1A certified by using the chemical shift of inorganic phosphate as an internal pH standard. The signal observed in Figure 1A employing freshly isolated troponin is therefore due to the presence of the bisphospho form within this mixture. The two remaining signals may be assigned to the two monophospho forms. Some of the isolated troponin complexes contain less than 1 mol of phosphate/mol of troponin I. Such a material has been characterized by Edman degradation of a phosphopeptide obtained by digestion of isolated troponin I with endoprotease Lys-C and trypsin [details of peptide isolation are described in Materials and Methods; compare Swiderek et al. (1988)]. From the three possible tryptic peptides the one comprising the phosphorylation domain in the N-terminus of cardiac troponin I ranging from amino acid 21 to 28 was isolated in homogeneous form. Prior to Edman degradation phosphoserine is converted to *S*-ethylcysteine (see Materials and Methods). Figure 2 shows that *S*-ethylcysteine originating from phosphoserine is detected only in cycle 4; serine is found in cycle 3. This demonstrates that only serine residue 24, but not serine 23, of troponin I is phosphorylated in this particular troponin preparation. Figure 1C shows the  $^{31}\text{P}$ -NMR spectrum of this troponin complex; one major signal can be detected at about 4.6 ppm. Again the pH of the sample is certified to be constant by employing the chemical shift of inorganic phosphate as internal pH standard. The corresponding signal at about 4.6 ppm in Figure 1A therefore originates from troponin I phosphorylated at serine 24. By exclusion, the third signal at about 4.4 ppm can then be assigned to the species phosphorylated at serine 23. All recorded signals representing these different species exhibit narrow line widths of about 3 Hz.

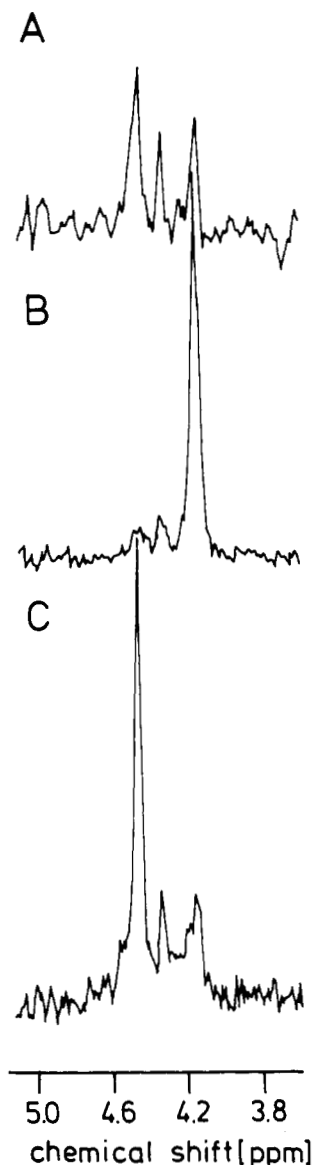


FIGURE 1:  $^{31}\text{P}$ -NMR spectra of cardiac holotroponin complexes of various degrees of phosphorylation. Sample preparation and NMR measurements are performed as described in Materials and Methods. Phosphate is bound exclusively to the I subunit. (For preparation of phosphotroponin I/dephosphotroponin T holocomplex see Materials and Methods.) The pH is constant in all three recorded spectra. Inorganic phosphate is used as internal pH reference. (A) Cardiac troponin (15 mg/mL) containing 1.4 mol of phosphate/mol of troponin I. (B) Cardiac troponin (20 mg/mL) containing 1.9 mol of phosphate/mol of troponin I after phosphorylation with cAMP-dependent protein kinase (see Materials and Methods). (C) Cardiac troponin (30 mg/mL) containing 0.6 mol of phosphate/mol of troponin I (for characterization of this material see Figure 2). In (A) 25 568, in (B) 23 808, and in (C) 21 240 scans were accumulated.

The observation of three signals (two for each monophospho and one for the bisphospho form) is a function of the integrity of the troponin complex. Isolation of cardiac troponin I from the holocomplex (see Materials and Methods) reduces the three-signal to a two-signal spectrum (compare Figure 3, panels A and B). Thus, either the two signals originating from each monophospho form merge at 4.4 ppm and the signal of the bisphospho form concomitantly shifts downfield to 4.3 ppm or the signal at 4.2 ppm originating at pH 6.8 from bisphosphotroponin I when present within the complex shifts downfield and splits into two signals which are observed at 4.3 and 4.4 ppm, respectively, i.e., at the positions where absorption of each monophospho form occurs in the holocomplex. Taking

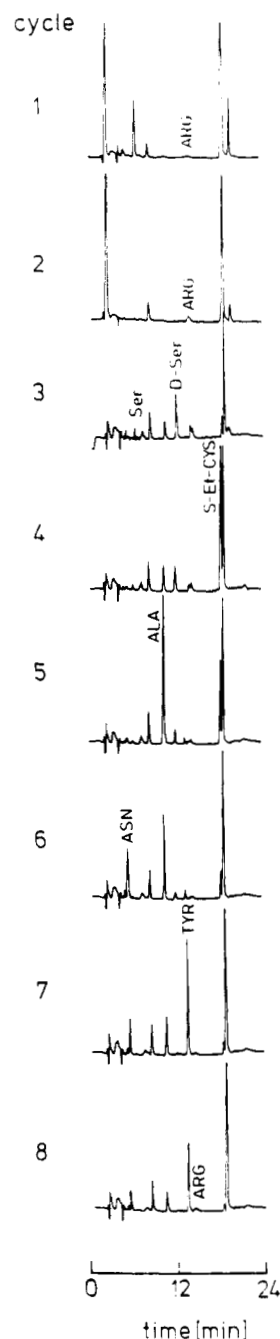


FIGURE 2: Localization of phosphoserine within the sequence of cardiac troponin I. Cardiac troponin containing 0.6 mol of phosphate/mol of troponin I was employed. Digestion with the endoprotease Lys C and trypsin, isolation of the phosphopeptide, and sequence analysis are performed as described in Materials and Methods. A 926-pmol portion of the purified tryptic phosphopeptide is applied onto the sequencer. The repetitive yield is 84.5%. The cycles shown represent positions 21–28 of cardiac troponin I. The occurrence of S-ethylcysteine in cycle 4 shows the position of the phosphoserine residue.

into account the results obtained with phosphopeptides as described below, the second conclusion is the most probable explanation.

The observation of a unique signal for the bisphospho form when present in the complex indicates that at least one of the other two troponin subunits, troponin T or troponin C, interacts with these phosphate groups. Therefore, the isolated troponin I was reconstituted with either isolated cardiac troponin T or cardiac troponin C. The proteins were solubilized in 6 M urea and 1.25 mM  $\text{CaCl}_2$ ; reconstitution is initiated by gradually removing urea. The formation of a 1:1 complex of

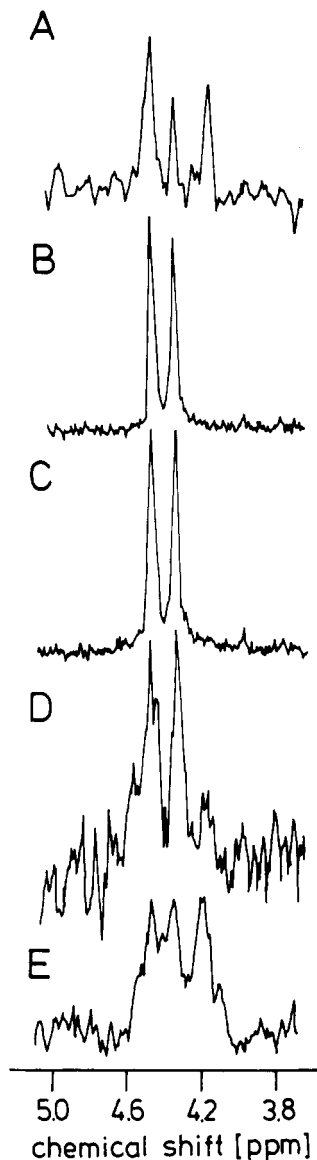


FIGURE 3:  $^{31}\text{P}$ -NMR spectra of holotroponin, isolated troponin I, and reconstituted complexes. NMR spectra are recorded as described in Materials and Methods. In each sample the pH is adjusted such that the chemical shift of inorganic phosphate is constant. All probes contained 1.4 mol of phosphate/mol of troponin I. (A) Cardiac troponin (15 mg/mL, 26 566 scans). (B) Cardiac troponin I (10 mg/mL) isolated from the material used in (A) (7932 scans). (C) Cardiac troponin I (20 mg/mL) reconstituted with cardiac troponin C in a 1:1 molar complex (18 172 scans). (D) Cardiac troponin I (12 mg/mL) reconstituted with cardiac troponin T in a 1:1 molar complex (34 368 scans). (E) Reconstituted cardiac holotroponin complex (T, I, C) in a 1:1:1 molar ratio (15 mg/mL, 45 248 scans).

troponin I with troponin C or T has been proven by gel filtration and analysis of the fractions by SDS gel electrophoresis and actomyosin ATPase activity tests (not shown). Figure 4C,D shows that neither the I/T nor the I/C complex reconstitutes the three-signal spectrum. Both complexes exhibit a two-signal  $^{31}\text{P}$ -NMR spectrum identical to that of isolated cardiac troponin I (compare Figure 3 panel B and panels C and D). However, reconstitution of the holotroponin complex from all three isolated subunits, cardiac troponin T, I, and C, reestablishes the original three-signal spectrum (compare Figure 3 panels A and E).

The three-signal spectrum shown in Figure 1A is recorded at neutral pH. Alkalinization of the buffer merges these signals until at pH > 8 only one signal is observed (Figure 4). Reacidification of the solution down to about pH 6 (the lowest

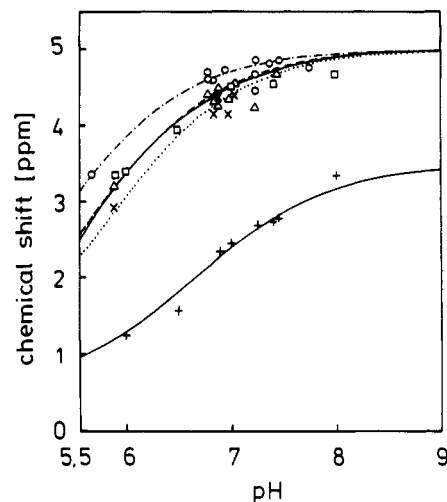


FIGURE 4: pH dependence of the  $^{31}\text{P}$ -NMR signals of cardiac holotroponin. NMR measurements are performed as described in Materials and Methods. Cardiac troponin, 20 mg/mL, containing about 1.4 mol of phosphate/mol of troponin I has been recorded at the pH value indicated. Inorganic phosphate (+) has been employed for internal standardization. The signal of phosphoserine at position 24 is shown by (O--O), that of phosphoserine at position 23 by (Δ--Δ), and that of the bisphospho form by (X--X). Free phosphoserine is shown as (□--□). Lines are calculated as described in Materials and Methods by a least-squares procedure.

pH possible without precipitation of the protein) reestablishes the three-signal spectrum; i.e., each phospho species (the two monophospho forms carrying phosphate at either serine 23 or serine 24 and the bisphospho form) exhibits different  $pK_a$  values. Extrapolation of the titration curves by a least-squares fitting of the data yields a  $pK_a$  value of ca. 5.3 and 5.6 for phosphoserine at positions 24 and 23, respectively. Apparently, the two phosphate groups present in the bisphospho form exhibit nearly identical  $pK_a$  values. These two phosphates, therefore, give rise to one single  $^{31}\text{P}$ -NMR signal exhibiting a  $pK_a$  value of about 5.8, which is higher than that of free phosphoserine. The  $pK_a$  value of phosphoserine 23 is very similar to that of noncovalently linked free phosphoserine ( $pK_a$  ca. 5.6, Figure 3).

A lower or a higher  $pK_a$  value of phosphoserine with the protein in comparison of free phosphoserine indicates interaction with basic or acidic amino acid residues, respectively. The high  $pK_a$  value of the bisphospho form is due to interaction with acidic groups of another troponin subunit as shown above. However, the difference in chemical shift of each monophosphorylated form must be due to interactions of phosphoserine with residues of cardiac troponin I itself. To narrow down the location of such (an) interacting amino acid(s) phosphopeptides were synthesized and their  $^{31}\text{P}$ -NMR spectra were recorded. At pH 7.0 also, a two-signal spectrum is obtained from the bisphosphorylated synthetic peptide PVRRRS(P)S-(P)ANYR representing the phosphorylation domain of troponin I (Figure 5). This demonstrates that even within this peptide the environment of each of the two phosphate groups is not identical. Again the chemical shifts are recorded as a function of pH and in comparison to free phosphoserine. Figure 6A shows that one of the phosphate groups exhibits a lower  $pK_a$  value than free phosphoserine ( $pK_a$  about 5.3) and the other one a  $pK_a$  of about 5.6, which corresponds to that of free phosphoserine. The assignment of each signal to one of the phosphate groups is possible by comparing the spectra of the corresponding monophospho forms. They were obtained by phosphorylating peptides where each of the two serine residues was replaced by an alanine residue (PVRRRSAANYR and

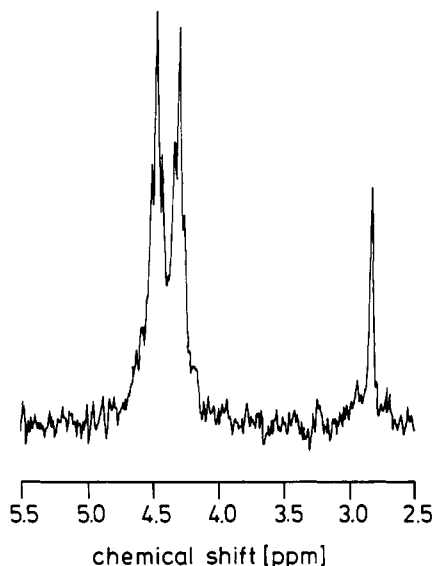


FIGURE 5:  $^{31}\text{P}$ -NMR spectrum of the bisphosphorylated synthetic peptide PVRRRS(P)S(P)ANYR. Synthesis, purification, phosphorylation, and NMR-measurements are performed as described in Materials and Methods. Peptide (2 mg) containing 2 mol of phosphate is solubilized in 700  $\mu\text{L}$  of buffer. The signal for inorganic phosphate as an internal standard is observed at 2.8 ppm. A total of 17 920 scans were accumulated.

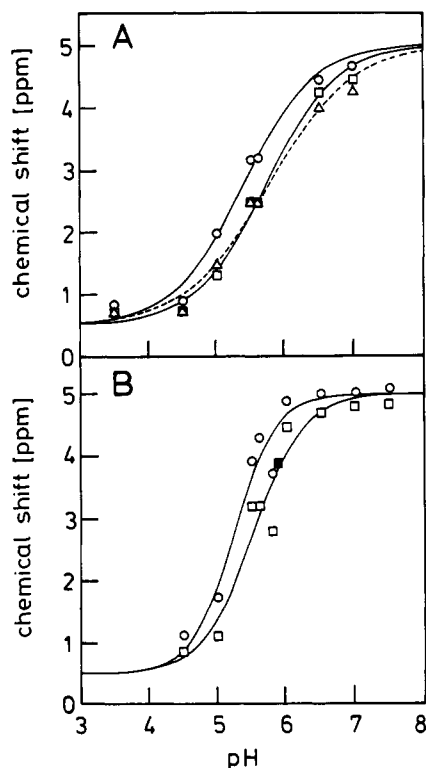


FIGURE 6: pH dependence of the  $^{31}\text{P}$ -NMR signals of synthetic peptides. (A) Titration curve of the bisphosphorylated synthetic peptide.  $\circ$  and  $\Delta$  correspond to the signals at 4.5 and 4.3 ppm in the spectrum of Figure 5, respectively. (B) Titration of the monophosphorylated peptides.  $\circ$  corresponds to PVRRRAS(P)ANYR and  $\blacksquare$  shows the chemical shift measured with PVRRRS(P)AANYR at pH 5.8. Free phosphoserine ( $\square$ ) was used as reference. The spectra are recorded as described in Materials and Methods.

PVRRRASANYR). As expected, each monophosphopeptide yields only one  $^{31}\text{P}$ -NMR signal (not shown). The monophosphorylated peptide containing phosphate bound to the serine equivalent to position 24 of troponin I [PVRRRAS(P)ANYR] yields a titration curve from which a  $\text{pK}_a$  value

of about 5.3 is calculated (Figure 6B). The phosphopeptide PVRRRS(P)AANYR was obtained in very limited amounts. Therefore only one spectrum could be recorded. At pH 5.8 it yields a signal exhibiting a chemical shift identical to that of free phosphoserine (Figure 6B).

## DISCUSSION

All three signals observed with cardiac holotroponin containing differently phosphorylated troponin I species exhibit a narrow line width. This indicates that the phosphate groups are not tightly bound but rather can rotate around the phosphoester bond. A tight binding results in line broadening due to a shortening of the relaxation time as has been observed in several proteins, e.g., in phosphorylase *a* or phosphorylase kinase. The phosphoserine at position 14 in glycogen phosphorylase *a* forms a salt bridge with Arg 69 and Arg 43' which is seen in the  $^{31}\text{P}$ -NMR spectrum as a downfield shift of the signal as well as by line broadening. A fast exchange between a tightly bound and a "free" form also causes an additional averaged downfield shift (Hörl et al., 1979). In phosphorylase kinase, probable hydrogen bonding of phosphoserine to another group occurs, resulting in an upfield shift of the signal as well as line broadening between 20 and 50 Hz (Kilimann et al., 1984). The relatively small line width of the signals obtained for troponin I in either form suggests that a strong binding—as hydrogen bonding—can be excluded.

Cardiac holotroponin containing differently phosphorylated troponin I species shows three  $^{31}\text{P}$ -NMR signals due to slightly different  $\text{pK}_a$  values of the respective phosphate groups. Only the bisphospho form within the holotroponin complex exhibits a higher  $\text{pK}_a$  value than free phosphoserine; the  $\text{pK}_a$  values of both phosphate groups are nearly identical. A higher  $\text{pK}_a$  value indicates a more acidic environment relative to the surrounding solute of free phosphoserine. This enhancement of  $\text{pK}_a$  values must clearly be due to the interactions of troponin I with either troponin T or C, as can be concluded from the disappearance of the bisphospho signal at 4.2 ppm by separating troponin I from the complex. Reconstitution with the T or C subunit should then reestablish the original spectrum. Even though the T and I as well as the C and I subunits easily form a complex, the N-terminal region of cardiac troponin I containing the two phosphate groups does not seem to be affected by this complex formation. Indeed, only the holotroponin complex provides the correct environment for the bisphosphorylated troponin I N-terminus. Thus it cannot be decided whether the phosphate groups within the bisphosphorylated troponin I interact with only one subunit, T or C, or with both of them.

Cardiac troponin I phosphorylation has been correlated to changes in  $\text{Ca}^{2+}$  affinity of troponin C (Holroyde et al., 1980), which indicates that this subunit interacts with the phosphorylation domain. Troponin C can also provide the acidic environment for the two phosphate groups as requested for the  $^{31}\text{P}$ -NMR signal of the bisphospho form. However, an interaction with troponin T showing large clusters of glutamic acids in its N-terminal region cannot be excluded. There are several possibilities how troponin C could influence the protonation degree of these two phosphate groups. First, in respect to the tertiary structure, one may assume that both phosphate groups are positioned as in the isolated bisphosphopeptide. Acidic groups of troponin C must then approach these phosphate groups in such a way that the protonation degree of each phosphate group is affected differently, which results in nearly identical  $\text{pK}_a$  values for both phosphates. Alternatively, binding of troponin C to troponin I may alter

the tertiary structure of the bisphosphorylated N-terminal region.

Even when present in the complex, the two monophospho forms of troponin I yield  $^{31}\text{P}$ -NMR signals which show chemical shifts identical to those obtained with isolated cardiac troponin I and with the synthetic bisphospho peptide comprising the phosphorylation domain. The  $pK_a$  value (5.3) of phosphoserine 24 is lower than that of free phosphoserine (5.6), indicating a basic environment. This basic amino acid must be provided by any positively charged amino acid within troponin I. However, it can be concluded that this interacting basic amino acid must be present within the phosphorylation domain itself, since in both synthetic peptides—PVRRRAS-(P)ANYR and PVRRRS(P)S(P)ANYR—the second phosphoserine, equivalent to phosphoserine 24, exhibits a  $pK_a$  value about 0.3 unit lower than that of free phosphoserine. Thus, this phosphate group must be near to one of the three preceding arginine residues.

Phosphoserine in position 23 [serine 6 within the peptides PVRRRS(P)S(P)ANYR and PVRRRS(P)AANYR] behaves like free phosphoserine; thus it is probably not involved in interactions. Also, in respect to this phosphate group the isolated troponin I and the synthetic phosphopeptides again behave identically.

On the basis of the specific interaction of the bisphospho form with another troponin subunit, probably troponin C, it is concluded that these two phosphate groups exert a specific function. In contrast, the two monophospho forms are apparently not involved in subunit interactions. Consequently, they might not have a specific function. A reasonable hypothesis explaining why two serine residues are phosphorylated by one protein kinase could be that the nonphosphorylated form exhibits an alternate function. Thus, in this instance phosphorylation creates a "three-state switch" which can only be generated with two phosphates; a singular phosphorylation can only create a "two-state switch".  $\text{Ca}^{2+}$ -binding measurements using the different phospho forms of troponin I as well as analysis of the *in vivo* phosphorylation state in muscle fibers will help in future to define such a multistate system.

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