Sequential phosphorylation of adjacent serine residues on the N-terminal region of cardiac troponin-I: structure–activity implications of ordered phosphorylation

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Abstract **We have used NMR spectroscopy to monitor the phosphorylation of a peptide corresponding to the N-terminal region of human cardiac troponin-I (residues 17–30), encompassing the two adjacent serine residues of the dual phosphorylation site. An ordered incorporation of phosphate catalysed by PKA was observed, with phosphorylation of Ser-24 preceding that of Ser-23. Diphosphorylation induced a conformational transition in this region, involving the specific association of the Arg-22 and Ser-24P side-chains, and maximally stabilised when both phosphoserines were in the di-anionic form. The results suggest that the second phosphorylation at Ser-23 of cardiac troponin-I is of particular significance in the mechanism by which adrenaline regulates the calcium sensitivity of the myofibrillar actomyosin Mg-ATPase.**

Key words: Cardiac troponin-I; Adrenaline; Protein phosphorylation; Serine kinase; NMR spectroscopy

1. Introduction

The phosphorylation of troponin-I that follows treatment of heart muscle with adrenaline reduces the sensitivity of the troponin-regulated cardiac actomyosin ATPase activity to calcium [1,2]. This effect is associated with the incorporation of up to 2 moles of phosphate at a PKA target site in the tissue-specific N-terminal region of troponin-I [3]. The target comprises adjacent serines [4]; residues 23 and 24 in the case of the human sequence [5]. The correlation between contractile response and the state of phosphorylation of these two serine residues is, however, currently ill-defined, and little is known about the structural basis of regulation by this dual-site phosphorylation.

Phosphorylation of both serine residues in vivo is considered to be catalysed by a single enzyme. As in the case of the insulin receptor protein-tyrosine kinase [6], this suggests some flexibility of the kinase active site to allow phosphorylation of both basal and monophosphorylated substrates. The target sequence (-IRRRSSNYR-) does not conform exactly to the consensus, single-site, recognition sequence of PKA, in which the site of phosphotransfer (P) is immediately preceded by a small residue (position P–1) and followed by a hydrophobic residue (P+1). We therefore undertook the real-time NMR study of the phosphorylation profile of a peptide encompassing residues 17–30 of human cardiac troponin-I, and investigated the localised structural consequences of multiple phosphorylation.

2. Materials and methods

The peptide (APIRRRSSNYRAYA), corresponding to residues 17–30 of the human cardiac troponin-I sequence, was obtained from Alta Bioscience, University of Birmingham. Its molecular weight and composition were verified by mass spectrometry and amino acid analysis. The peptide and its phosphorylated derivates were purified by HPLC, using a Vydac C_{18} column and a water/acetonitrile gradient. Phosphorylation was carried out with bovine heart PKA (Sigma, Poole, UK) at 27°C in the presence of 50 mM Tris, 1 mM $MgCl_2$, 1 mM cAMP and 5 mM ATP in H_2O or ^2H_2O, pH 7.6. For gel analysis, samples were removed at intervals and electrophoresed through 15% Acrylogel 5 gels (BDH, Poole, UK) in the presence of 5% acetic acid, 6 M urea. Gels were fixed with 1.5% perchloric acid, stained with Coomassie blue R250, and immediately photographed as the bands faded rapidly. NMR experiments were performed using a Bruker AMX500 spectrometer. Standard pulse sequences were used for 2D ^1H homonuclear TOCSY and NOESY experiments. Water suppression was achieved by presaturation during the relaxation delay or by the Watergate gradient pulse scheme [7].

3. Results

3.1. Ordered phosphorylation of the adjacent serine residues

Phosphorylation of the cardiac troponin-I peptide by PKA could be readily monitored on acid gels due to the altered mobility of the phosphorylated species with increasing negative charge. Conversion of the peptide to a monophosphorylated form and subsequent diphosphorylation are illustrated in Fig. 1. As this procedure could not identify the serine residue(s) modified in the monophosphorylated species, we used ^1H NMR spectroscopy to visualise the phosphorylation profile in real time and identify the phosphorylated serine(s) at each stage.

Two distinct sets of spectral changes were apparent during the phosphorylation time-course (Fig. 2), indicating that phosphate incorporation occurred in successive stages. The first stage of phosphorylation was associated with alterations in the resonances of Ser-24 and Tyr-26, while the signals of Ser-23 remained unperturbed (Figs. 2 and 3). HPLC analysis of phosphorylation reactions halted at this stage revealed only a single major species, indentified by 2D NMR methods as peptide monophosphorylated on Ser-24 (Fig. 3). Thus, the initial phosphorylation occurred exclusively on Ser-24, and reached >90% completion before the second stage began. Peptide monophosphorylated on Ser-23 was never seen.

The second stage of phosphorylation was characterised by shifts in the resonances of Ser-23 and Asn-25 (Figs. 2 and 3),

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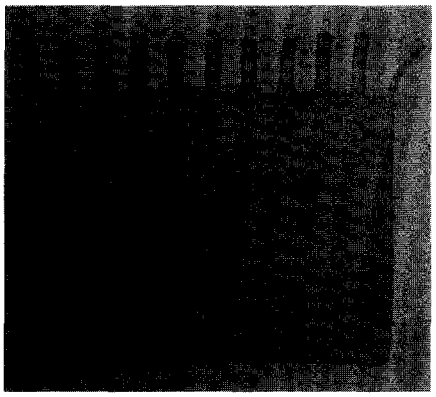


Fig. 1. Timecourse of troponin-I peptide phosphorylation monitored by acid gel electrophoresis. Conditions were as described in section 2. Samples (tracks 1–7) were taken 0, 5, 15, 30, 60, 90 and 120 min, respectively, after addition of PKA.

while the signals of Ser-24P were relatively unperturbed. HPLC analysis revealed a major species, deduced from its NMR spectrum to be phosphorylated on both Ser-23 and Ser-24, along with small amounts (< 10%) of the Ser-24-monophosphorylated peptide. This stage was 3–4 times slower than the first, and clearly represented the further phosphorylation at Ser-23 of the Ser-24-monophosphorylated troponin-I peptide.

3.2. Conformational consequences of sequential phosphorylation

To confirm the identity of the phosphorylated serine residues at each stage of the phosphorylation process and to investigate any resulting conformational effects, 2D ^{1}H homonuclear NOESY studies of the isolated peptides were undertaken. The fingerprint (backbone-NH/C_αH) regions of the spectra of the basal and monophosphorylated species are compared in Fig. 3, along with the corresponding 2D ^{1}H TOCSY spectra. The downfield shift of the Ser-24-NH and $C_\beta H_2$ proton resonances identify it unequivocally as the residue modified upon monophosphorylation, an assignment also supported by the spectral changes shown by these resonances when the solution pH was titrated through the pK_a of the phosphate group (data not shown). Interestingly, the $C_\delta H_2$ (Fig. 2) and side-chain -NH protons of Arg-22 (visualised in H_2O, pH 5.7: data not shown) were both shifted downfield in the monophosphorylated species, the latter by around 0.3 ppm. This suggested a specific interaction between this arginine side-chain and the phosphate group of Ser-24P. Further evidence for a structural adjustment and restriction of the conformational averaging of the sequence around Ser-24 upon monophosphorylation was obtained from the increased number and strength of through-space (NOE) interactions (Figs. 3 and 4). The extent and intensity of NOE interactions were further increased upon diphosphorylation (Fig. 4), indicating that the adjacent phosphorylated residues imposed further conformational restrictions on the surrounding sequence.

To investigate the contribution of the charge of the phosphate groups to this structural stabilisation, NOESY spectra of the diphosphorylated peptide were obtained at pH 5.6 and pH 7.0. Despite an overall reduction in NOE intensities in the fingerprint region at the higher pH (resulting from increased -NH exchange rates with the H_2O protons), a further group of NOE cross-peaks became apparent at pH 7.0, most notably those between Arg-22 and Ser-24P (Fig. 4). These cross-peaks were not present in a NOESY spectrum of the monophosphorylated peptide at pH 7.0 (data not shown). Stabilisation of the association between the Arg-22 and Ser-24P side-chains is thus favoured when the phosphate groups are in the di-anionic form.

4. Discussion

When isolated from perfused heart, troponin-I contains about 1 mol phosphate/mol protein, located mainly at its N-terminal region, which increases to 2 mol/mol after intervention with adrenaline [3]. Associated with the increased phosphorylation is a fall in the calcium sensitivity of the myofibrillar actomyosin Mg-ATPase. Although the order of phosphorylation of the N-terminal site in vivo remains undetermined, our results suggest that phosphorylation of Ser-24 precedes that of Ser-23. The PKA consensus for the P + 1 and P–1 positions is matched more closely by the Ser/Asn flanking Ser-24 than by the

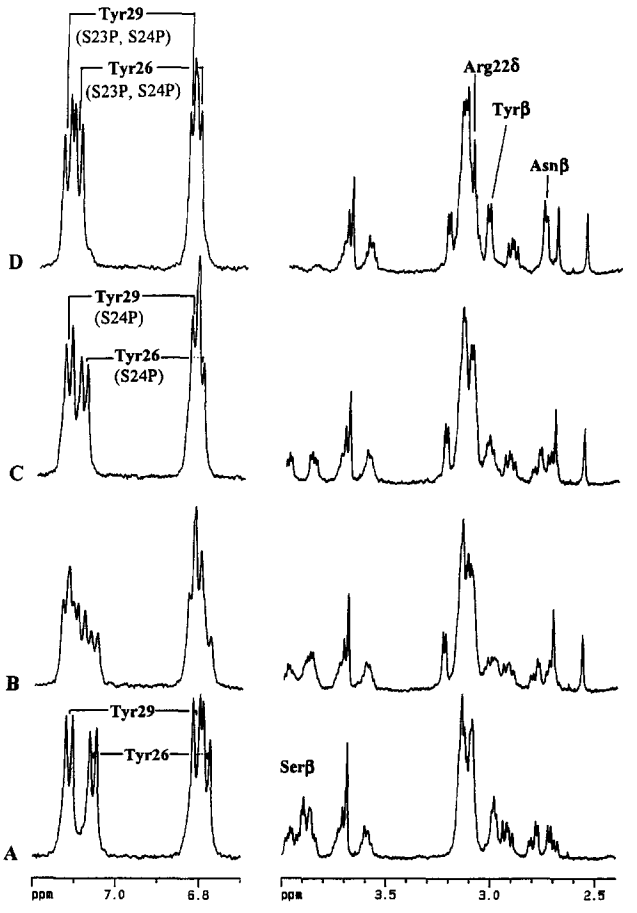


Fig. 2. Time course of troponin-I peptide phosphorylation monitored by ^{1}H NMR spectroscopy. Spectra (A–D) were summed from 32 transients (total acquisition time 1.5 min), obtained immediately before, then 15, 34 and 120 min after initiation of phosphorylation.

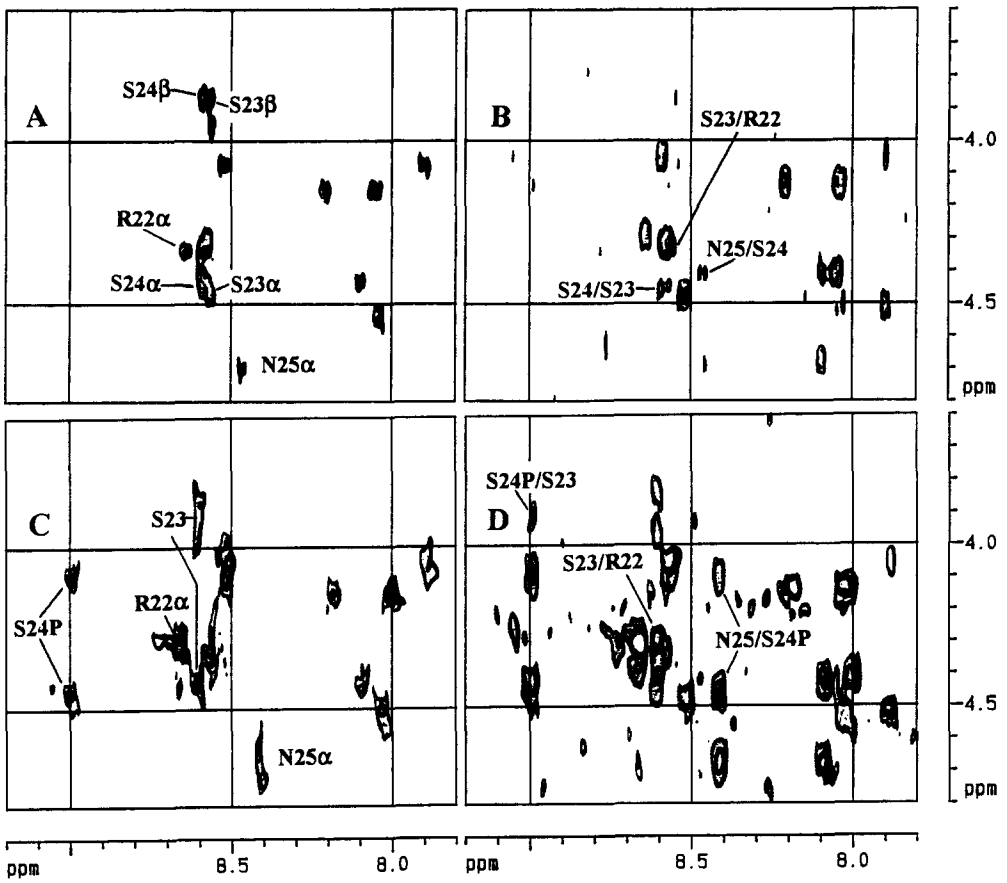
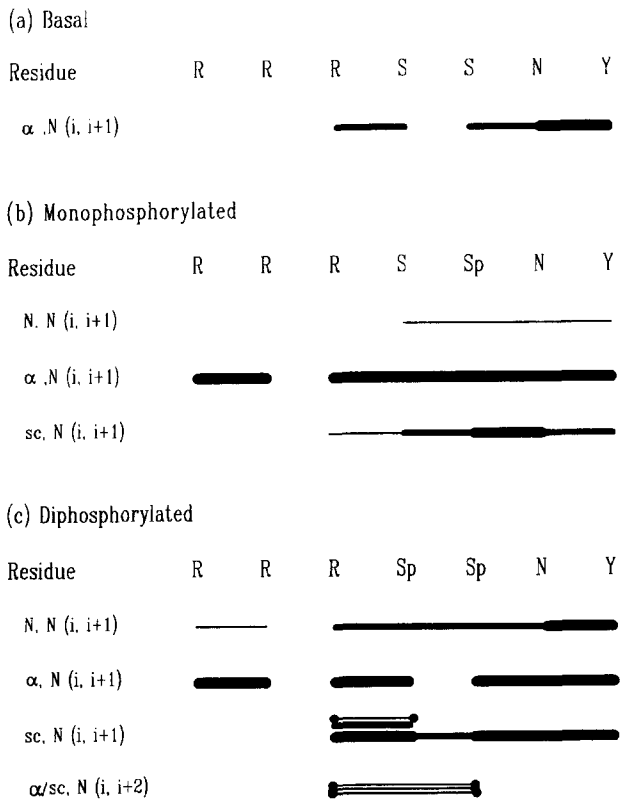


Fig. 3. 2D ^{1}H homonuclear NMR spectra of basal and Ser-24P-monophosphorylated troponin-I peptide. (A) basal peptide TOCSY; (B) basal peptide NOESY; (C) Ser-24P peptide TOCSY; (D) Ser-24P peptide NOESY. TOCSY spectra were acquired in 90% H_2O, pH 5.6, at 12°C with a mixing time of 60 ms. NOESY spectra were acquired under identical conditions, with a mixing time of 600 ms.



Arg/Ser around Ser-23. In particular, the preference for a small residue at P–1 is consistent with the docking mode of the inhibitor peptide, PKI, to PKA, in which this side-chain nestles against the nucleotide-binding, glycine-rich loop [9]. This analysis suggests that the main N-terminal residue phosphorylated in the normal perfused heart would be Ser-24, with the decrease in calcium sensitivity following adrenaline intervention arising from the subsequent phosphorylation of Ser-23. However, while it is widely accepted that PKA catalyses the phosphorylation of both N-terminal serines in vivo, the fact that phosphorylation of troponin-I occurs while it is part of the troponin complex may allow steric influences to cause deviation from this pattern. Endogenous phosphatase activity may also come into play. For example, if both Ser-23 and Ser-24-monophosphorylated substrates were available in vivo, our analysis

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Fig. 4. Partial inter-residue NOE maps of basal, monophosphorylated and diphosphorylated troponin-I peptides. Strong, medium and weak negative NOE cross-peaks are indicated by ■, ▬, and —, respectively. NOE peaks observed at pH 7.0 but not at pH 5.6 are indicated by ●——●. N = backbone-NH; α = -C_αH; SC = side-chain (all other protons). The NOE interaction between -C_αH of S23P and -NH of S24P in the diphosphorylated peptide could not be assigned unambiguously due to spectral overlap.

predicts that further phosphorylation of the Ser-24P species would be favoured, given the bulky, di-anionic nature of the P–1 position for the Ser-23P species.

Diphosphorylation of the cardiac troponin-I peptide is shown to result in a well-defined organisation about the dual phosphorylation site, involving the stabilisation of a specific interaction between Arg-22 and Ser-24P. Such a change in vivo may cause the loosening of any association of the N-terminal region of troponin-I with other proteins within the cardiac thin filament assembly, and/or the cooperative modification of other troponin-I contacts. This may explain the observations that diphosphorylation of troponin-I causes a reduction in affinity and cooperatively in its binding to F-actin [8], and a decrease in its affinity for troponin-C [10].

Diphosphorylation may mediate the regulation of actomyosin ATPase activity in smooth muscle, where ATPase activity is increased upon phosphorylation of myosin regulatory light chain at Ser-19 and further enhanced by additional phosphorylation at Thr-18 [11]. Mutation of both Ser-19 and Thr-18 to Asp gave only partial ATPase activation [11]. This may be explained by the single negative charge of the aspartate side-chain being less effective than the doubly-charged phosphate; in the cardiac troponin-I system, the conformational transition observed upon diphosphorylation was more effectively stabilised by doubly-charged phosphates. The region of the myosin regulatory light chain preceding the dual phosphorylation site, Arg-13–Arg-16, is required for the phosphorylation mediated regulation. The role of these basic residues may be to interact with the nearby phosphorylated side-chains in a similar manner to the Arg-22/Ser-24P interaction of troponin-I.

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