The Sites of Phosphorylation of Rabbit Cardiac Troponin I by Adenosine 3':5'-Cyclic Monophosphate-Dependent Protein Kinase

EFFECT OF INTERACTION WITH TROPONIN C

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1. Troponin I prepared from rabbit hearts contains 1.0–1.5 mol of P/mol when isolated by affinity chromatography. Most of the covalently bound phosphate is located in residues 1–48 of the molecule. 2. 3':5'-Cyclic AMP-dependent protein kinase catalyses phosphorylation at serine-20 and serine-146. Serine-20 is more rapidly phosphorylated than serine-146. 3. In troponin I prepared from frozen hearts by affinity chromatography about 0.3–0.5 mol of P/mol is associated with serine-20 and 0.8–1.0 mol of P/mol with other site(s) in residues 1–48 of the molecule. 4. Phosphorylation at serine-20 and serine-146 is not significantly inhibited by troponin C. 5. The mechansim of the interaction of troponin C with cardiac troponin I is discussed in the light of these results.

It is now well established that troponin I of fast skeletal muscle has sites that are specific for phosphorylation catalysed by 3':5'-cyclic AMP-dependent protein kinase and phosphorylase kinase (Moir *et al.*, 1974; Huang *et al.*, 1974; Perry & Cole, 1974). The phosphorylation of troponin I from cardiac muscle is likewise catalysed by both enzymes, but with 3':5'-cyclic AMP-dependent protein kinase the rate of phosphorylation *in vitro* is about 30 times faster than that obtained under the same conditions with skeletal troponin I isolated from fast skeletal muscle (Cole & Perry, 1975). This observation suggests that the phosphorylation of troponin I catalysed by 3':5'-cyclic AMP-dependent protein kinase, an enzyme that has been implicated in the regulation of a wide range of cellular processes, may be of special significance for cardiac function. The observation (Cole & Perry, 1975) that cardiac troponin I contains larger amounts of covalently bound phosphorus than does skeletal-muscle troponin I when isolated rapidly from fresh tissue also supports this view. The implication of these studies *in vitro* is further strengthened by the demonstration that in the perfused heart the extent of phosphorylation of troponin I rises when the force increases after treatment with adrenaline (England, 1975; Solaro *et al.*, 1976).

The extent of phosphorylation of troponin I *in vivo* will depend on the effects of interaction with other components of the troponin complex. For example, the phosphorylation of fast-skeletal-muscle troponin I by both kinases is markedly inhibited in the presence of troponin C (Perry & Cole, 1974), owing to complex-formation (Head & Perry, 1974), which leads to masking of the phosphorylation sites by a process not yet fully understood, although possible mechanisms

have been suggested (Perry *et al.*, 1976). Although cardiac troponin I forms complexes with both the cardiac and skeletal-muscle forms of troponin C (Syska *et al.*, 1974; Head *et al.*, 1977), some differences in the nature of the interaction with troponin C exist, compared with that occurring in fast-skeletal-muscle troponin I. Whereas the phosphorylation of cardiac troponin I catalysed by phosphorylase kinase is inhibited by troponin C, this is not the case with 3':5'-cyclic AMP-dependent protein kinase.

As part of a general investigation of the role of troponin I in cardiac function we have investigated the nature and location of the sites on cardiac troponin I specific for phosphorylation catalysed by 3':5'-cyclic AMP-dependent protein kinase. An aim of the present study is to examine the mode of interaction of troponin I with troponin C and to relate the role of phosphorylation of troponin I to the function of the troponin complex in cardiac muscle. Our studies indicate that cardiac troponin I possesses a unique phosphorylation site not present in the skeletal forms of troponin I. Phosphorylation of this site occurs *in vivo* and is not inhibited by troponin C.

Some aspects of this work have been briefly reported (Solaro *et al.*, 1976; Cole *et al.*, 1976).

Materials and Methods

Materials

Bovine cardiac 3':5'-cyclic AMP-dependent protein kinase (type 1) was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Trypsin (1-chloro-4-phenyl-3-L-tosyl-amidobutan-2-one-treated) was from Cambrian Chemicals, Croydon, Surrey CR0 4XB, U.K.

[γ-^{32}P]ATP was purchased as the freeze-dried ammonium salt from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of troponin and components

Troponin was prepared from rabbit skeletal muscle by the method of Ebashi *et al.* (1971) and from frozen rabbit hearts obtained from a commercial supplier (Buxted Rabbit Co., Buxted, Sussex, U.K.) by the method of Tsukui & Ebashi (1973). Both preparations were fractionated into troponin I, troponin T and troponin C by chromatography on DEAE-cellulose (Perry & Cole, 1974). Troponin I was also isolated directly from frozen rabbit hearts by the affinity-chromatographic method of Syska *et al.* (1974).

Incubation with bovine cardiac protein kinase

Bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase (50–100 μg/ml) was incubated with either troponin I (0.5–4.0 mg/ml) or the peptides (0.5–1.0 mg/ml) derived from CNBr digestion of troponin I, under the conditions specified by Cole & Perry (1975). The enzymic reactions were stopped by the addition of an equal volume of ice-cold 15% (w/v) trichloroacetic acid and the precipitated protein was washed four times with 3 ml of cold 7.5% (w/v) trichloroacetic acid. When the protein was required for further digestion, the enzymic reaction was stopped by the addition of solid guanidine hydrochloride to a final concentration of approx. 6 M and the protein isolated by gel filtration on Sephadex G-75 in 10 mM-HCl as described by Moir *et al.* (1977). In those experiments in which the troponin I was phosphorylated in the presence of troponin C the latter procedure was used to stop the reaction. On gel filtration under these conditions the troponin I was eluted first, well separated from troponin C.

Determination of phosphate and protein

Total covalently bound phosphate was determined on troponin I preparations that had been washed five times with cold 5% (w/v) trichloroacetic acid and the fractions obtained from such samples after digestion with CNBr or proteolytic enzymes. H_2SO_4 (5 M; 0.5 ml) was added to troponin I (1–3 mg) or up to 2.0 ml fractions of digests and heated for 2–3 h at 160°C. After cooling, 0.15 ml of phosphate-free 100-vol. H_2O_2 was added and the samples were heated at 160°C for a further 2 h. Water (either 4.4 ml or 2.5 ml) was added, and 10% of the sample removed for nitrogen determination (Strauch, 1965). The phosphate content of the remaining 90% was measured by the method of Bartlett (1959) with 0.2 ml of 5% (w/v) ammonium molybdate and 0.2 ml of reducer for each assay. ^{32}P was determined by the

Čerenkov method (Gould *et al.*, 1972) as described by Perry & Cole (1974).

Digestion of ^{32}P-labelled troponin I with CNBr

Troponin I was digested with CNBr in 0.1 M-HCl as described previously (Moir *et al.*, 1977). The digests were freeze-dried without the addition of water and then dissolved (10–30 mg/ml) in 6 M-guanidine hydrochloride/0.4 M-Tris/0.2 M-HCl (pH 8.0)/1 mM-dithiothreitol and the cysteine residues were carboxymethylated by using iodoacetic acid as described by Moir *et al.* (1974) before fractionation by gel filtration as described below.

Fractionation and analysis of peptides

The CNBr peptides were fractionated on a column (2.5 cm × 110 cm) of Sephadex G-75 equilibrated with 0.01 M-HCl. Peptides were eluted in 5 ml fractions and were located in the eluate by monitoring the A_{215} or A_{230}. The distribution of ^{32}P in the eluate was determined by measuring the radioactivity in every second 5 ml fraction by the Čerenkov procedure (Gould *et al.*, 1972). Two CNBr peptides (peptides CCN3 and CCN4 in Fig. 1) were incompletely resolved by this procedure; they were purified by further chromatography on a column (3.0 cm × 210 cm) of Sephadex G-50 in 0.01 M-HCl as described by Grand *et al.* (1976), whose nomenclature for the CNBr peptides has been used throughout (Fig. 1).

Enzymic digestion of peptides

^{32}P-labelled CNBr peptides were digested with trypsin in 1% NH_4HCO_3, pH 7.9, as described by Moir *et al.* (1977). Tryptic digests were fractionated by gel filtration on a column (2.0 cm × 110 cm) of Sephadex G-25 equilibrated with either 0.01 M-HCl or 0.05 M-NH_4HCO_3 and peptides containing radioactivity were further purified by high-voltage electrophoresis at pH 6.5, pH 2.0 and pH 3.5 as described by Wilkinson (1974).

Detection of peptides by radioautography, elution of peptides, amino acid analysis and *N*-terminal analysis by dansylation were performed by previously described methods (Moir *et al.*, 1977).

Results

Distribution of phosphate in the CNBr peptides of cardiac troponin I

The covalently bound phosphate content of cardiac troponin I depends on the method of preparation and the history of the hearts used for the preparation (Cole & Perry, 1975; Solaro *et al.*, 1976). When isolated from the troponin complex prepared from
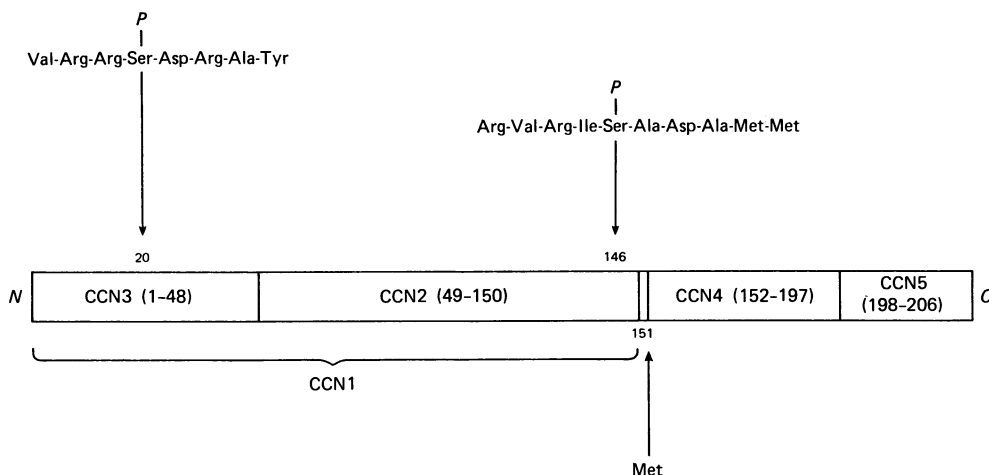
Fig. 1. *Representation of the primary sequence of rabbit cardiac troponin I, indicating the relative positions of the CNBr peptides CCN1, CCN2, CCN3, CCN4 and CCN5 (Grand et al., 1976) and the phosphorylation sites specific for 3′:5′-cyclic AMP-dependent protein kinase*
Numbers are residue positions in the sequence. N, N-Terminus; C, C-terminus.

frozen rabbit hearts purchased from a commercial supplier by the aqueous extraction procedure of Tsukui & Ebashi (1973) the phosphate contents were low (approx. 0.5 mol of P/mol). Most of the preparations used in the present investigation were obtained by affinity chromatography from frozen rabbit hearts and had phosphate contents in the range 1.0–1.5 mol of P/mol of troponin I.

Phosphate added by 3′:5′-cyclic AMP-dependent kinase. After incubation for 30 min at 30°C with 3′:5′-cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP the phosphate content of rabbit cardiac troponin I that had been isolated by affinity chromatography was increased by 0.2–0.5 mol of P/mol. Gel filtration on Sephadex G-75 of the CNBr digest of troponin I labelled with ^{32}P in this way yielded the elution profile illustrated in Fig. 2 (result of one experiment, which was typical of the twelve experiments performed). Usually five major peaks of 230 nm-absorbing material and three major peaks of radioactivity were obtained. Two small peaks of radioactivity at the front of the eluate (between 110 and 140 ml in Fig. 2) were shown by polyacrylamide-gel electrophoresis in 6 M-urea at pH 3.2 (Panyim & Chalkley, 1969) to contain large peptide fragments resulting from incomplete cleavage at the methionine residues of troponin I. Fraction A, obtained by pooling the tubes indicated (Fig. 2), migrated as a single radioactive band on polyacrylamide-gel electrophoresis at pH 3.2. From the amino acid analysis (Table 1) of this peptide and its position of elution on gel filtration it was concluded that it was

identical with peptide CCN1 isolated by Grand *et al.* (1976) from CNBr digests of rabbit cardiac troponin I and which has been shown by these workers to be a partial digestion product consisting of residues 1–150 (Fig. 1). Polyacrylamide-gel electrophoresis of fraction B (Fig. 2) indicated that it also consisted of a single radioactive peptide, which from its amino acid content (Table 1) was shown to be identical with peptide CCN2, likewise isolated by Grand *et al.* (1976) from rabbit cardiac troponin I.

Fraction C (Fig. 2) was shown by polyacrylamide-gel electrophoresis at pH 3.2 to contain two components that could be separated, by gel filtration on Sephadex G-50, into peptide C1, which contained all the radioactivity, and peptide C2, which was not radioactively labelled (Fig. 3). From their amino acid compositions (Table 1) it was concluded that peptides C1 and C2 corresponded respectively to peptide CCN3 (residues 1–48) and peptide CCN4 (residues 152–197) isolated by Grand *et al.* (1976) from CNBr digests of rabbit cardiac troponin I.

These results show that the ^{32}P transferred to cardiac troponin I by the action of 3′:5′-cyclic AMP-dependent protein kinase was virtually all located in the region of the molecule consisting of residues 1–150.

Endogenous phosphate. To determine the location of the endogenous phosphate in the protein, a CNBr digest of troponin I prepared directly from frozen rabbit heart muscle by affinity chromatography was fractionated on Sephadex G-75, and total phosphate analysis carried out on the eluted fractions. In the
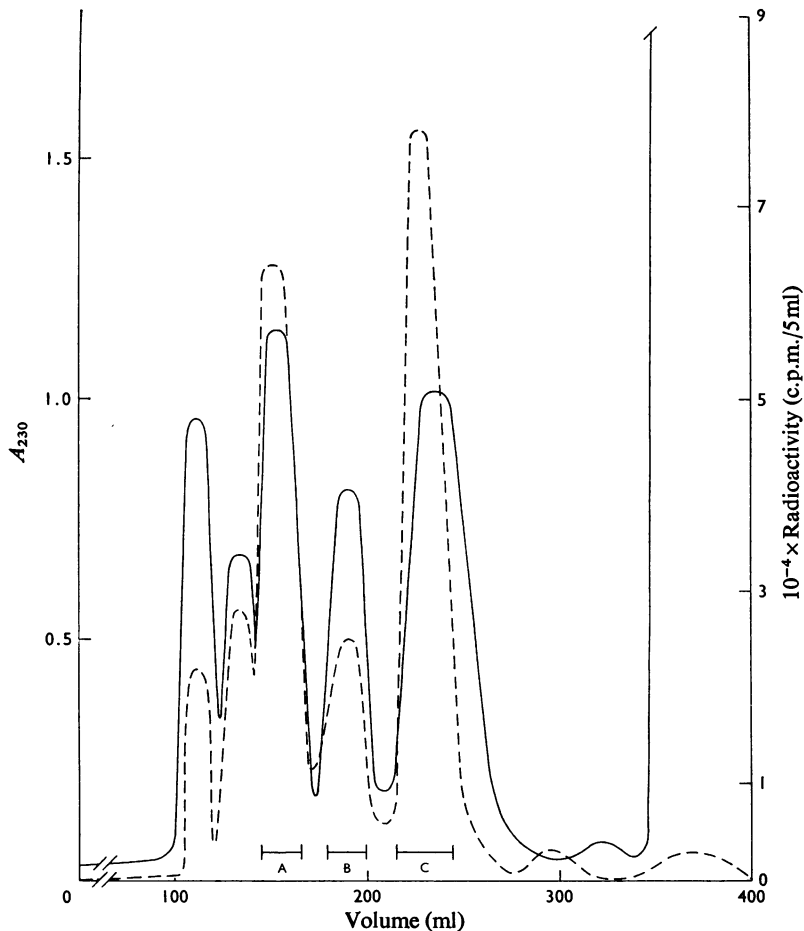
Fig. 2. *Gel filtration of a CNBr digest of ^{32}P-labelled cardiac troponin I*

A carboxymethylated CNBr digest of ^{32}P-labelled troponin I (approx. 50 mg) prepared as described in the Materials and Methods section and dissolved in 4.0 ml of 6 M-guanidine hydrochloride/0.4 M-Tris (adjusted to pH 8.0 with 1.0 M-HCl)/ 1 mM-dithiothreitol was applied to a column (2.5 cm × 110 cm) of Sephadex G-75 equilibrated and eluted with 0.01 M-HCl. Fractions (5 ml) were collected, the A_{230} of each fraction was measured and radioactivity measured in alternate fractions. Samples were pooled as fractions A, B and C as indicated by the horizontal bars. ———, A_{230}; ————, radioactivity.

experiment illustrated in Fig. 4 about 90% of the phosphate applied to the column in the CNBr digest was recovered in the eluate. Most of the recovered phosphate was present in fraction A, containing peptide CCN1 (residues 1–150), and in fraction C, containing peptides CCN3 (residues 1–48) and CCN4 (residues 152–197). From the distribution of phosphate in the eluate obtained by gel filtration of fraction C on Sephadex G-50 it was apparent that at least 90% of the total phosphate present in fraction C was located in peptide CCN3.

These results indicated that almost all the endogenous phosphate present in cardiac troponin I is

located in the region consisting of the first 48 residues of the molecule.

Stability of phosphate covalently bound to troponin I. The phosphate bound to troponin I was stable under the conditions of CNBr cleavage, which was carried out in 0.1 M-HCl at 20°C. For example, it was shown in the experiment illustrated in Fig. 4 that 90% of the phosphate present in the protein isolated by affinity chromatography could be recovered covalently linked to the fractionated CNBr peptides. Further, the additional phosphate incorporated after incubation with [γ-^{32}P]ATP and 3′:5′-cyclic AMP-dependent protein kinase was also stable on digestion by CNBr

Table 1. *Amino acid composition of the peptides obtained by CNBr digestion of ^{32}P-labelled cardiac troponin I*

Rabbit cardiac troponin I that had been phosphorylated with 3′:5′-cyclic AMP-dependent protein kinase and [γ-^{32}P]-ATP was digested as described in the Materials and Methods section. The peptides were fractionated as described in Figs. 2 and 3 and in the text. The values are the averages for two or three preparations. The numbers in parentheses are the peptide compositions reported by Grand *et al.* (1976). See Fig. 1 for peptide nomenclature. —, Residue absent.

	Composition of peptide (mol/mol of peptide)			
	CCN1	CCN2	CCN3	CCN4
Asp	12.3 (12.0)	8.7 (8.8)	4.1 (3.4)	4.7 (6.3)
Thr	7.4 (7.4)	4.8 (5.2)	2.3 (2.0)	3.0 (2.6)
Ser	6.8 (6.5)	3.4 (2.6)	3.5 (4.0)	1.4 (0.9)
Glu	22.3 (21.2)	15.6 (16.1)	5.7 (4.2)	7.2 (7.1)
Pro	5.4 (5.5)	2.5 (2.2)	2.8 (2.6)	— —
Gly	8.3 (8.4)	5.6 (5.5)	2.2 (1.6)	2.9 (2.9)
Ala	18.4 (18.9)	9.9 (10.0)	7.1 (8.7)	4.0 (3.2)
Cys*	† †	† †	— —	— —
Val	7.1 (7.1)	5.5 (4.6)	1.8 (1.3)	2.0 (1.7)
Met	0.6 †	— —	— —	— —
Ile	5.8 (6.1)	4.2 (5.0)	1.3 (1.0)	1.1 (1.0)
Leu	15.7 (16.8)	10.8 (12.2)	4.1 (3.4)	6.1 (6.7)
Tyr	2.4 (2.6)	1.1 (1.0)	1.2 (1.1)	— —
Phe	3.0 (3.1)	2.7 (3.0)	0.5 —	— —
His	1.9 (2.5)	1.2 (1.1)	0.9 (0.9)	0.9 (1.0)
Lys	13.3 (14.3)	8.3 (8.7)	6.0 (5.8)	5.1 (6.6)
Arg	17.1 (17.6)	10.6 (12.1)	5.4 (5.9)	3.9 (4.0)
Hse	† †	† †	† †	† †
^{32}P incorporated	0.32	0.125	0.16	0

* Identified as *S*-carboxymethylcysteine.

† Residue was identified as homoserine, but could not be measured.

under similar conditions. Fig. 2 shows that less than 5% of the ^{32}P in the digest was present as P$_i$, eluted between 340 and 400 ml. It was therefore concluded that the phosphate distribution in the troponin I was not significantly affected by the acidic conditions used for CNBr digestion.

Identification of residues phosphorylated by 3′:5′-cyclic AMP-dependent protein kinase

When troponin I was incubated with [γ-^{32}P]ATP and 3′:5′-cyclic AMP-dependent protein kinase, about 75% of the radioactivity recovered after fractionation of the CNBr peptides was present in peptide CCN3 (Fig. 2). This implied that the major site(s) of the phosphorylation catalysed by this enzyme were located in the region represented by residues 1–48. When a tryptic digest of ^{32}P-labelled peptide CCN3 (see the Materials and Methods section) was dissolved in 50 mM-NH$_4$HCO$_3$, pH 7.9, and subjected to gel filtration on Sephadex G-25 equilibrated against the same buffer, it separated into three or four peaks of 215 nm-absorbing material. The first peak eluted was radioactive, and, if the material of which it was composed was fractionated by paper electrophoresis at pH 6.5, a complex pattern, consisting of at least 13 bands, was obtained. Four of the bands were radioactive, and, when these were

further purified by paper electrophoresis at pH 2.0, 97% of the total radioactivity recovered was shown to be present in two of them, peptides CCN3TA and CCN3TB (Table 2). The minor radioactive bands were not further investigated. The total radioactivity recovered in these four peptides was 60–65% of that applied to the gel-filtration column. From their amino acid compositions (Table 2) peptides CCN3TA and CCN3TB must be derived from the same region of peptide CCN3, for both contained tyrosine, only one residue of which (at position 24) is present in peptide CCN3. Both peptides also contained a single serine residue, and the N-terminus of peptide CCN3TA was shown to be arginine. It was therefore concluded that the serine residue at position 20 in the sequence reported by Grand *et al.* (1976) was the major phosphorylation site (see Fig. 1). The sequence in the immediate vicinity of the phosphorylation site is Arg-Ser-Asx-Arg-Ala-Tyr.

The mobilities at pH 6.5 of peptides CCN3TA and CCN3TB purified in various experiments during the course of the work were variable. The mobilities of these peptides suggested that residue 21 was asparagine, although on one occasion the mobilities of peptides CCN3TA and CCN3TB were consistent with residue 21 being aspartic acid, as reported by Grand *et al.* (1976). The reasons for this variability have not been further investigated.
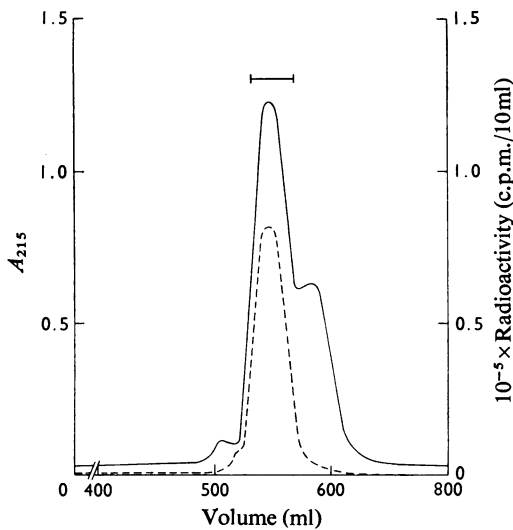
Fig. 3. *Gel filtration of fraction C from the CNBr digest of cardiac troponin I*

Fraction C obtained by gel filtration of a CNBr digest of ^{32}P-labelled troponin I on Sephadex G-75 (Fig. 2) was freeze-dried and redissolved in 3.0 ml of 6 M-guanidine hydrochloride/0.4 M-Tris (adjusted to pH 8.0 with 1.0 M-HCl) and applied to a column (3.0 cm × 210 cm) of Sephadex G-50 equilibrated with 0.01 M-HCl. Fractions (10 ml) were collected. The horizontal bar indicates the fraction that was pooled for subsequent digestion with trypsin. A duplicate experiment gave the same result. ——, A_{215}; – – – –, radioactivity.

When isolated by gel filtration, peptide CCN3 is a mixture of the phosphorylated and dephosphorylated forms. The ^{32}P-containing tryptic peptides derived from it, CCN3TA and CCN3TB, were isolated by paper electrophoresis and therefore consist only of the phosphorylated forms. By comparing the specific radioactivities of peptide CCN3 with those of the tryptic peptides derived from it, it was possible to estimate the extent of phosphorylation of serine-20 before incubation with kinase, as virtually all the phosphorylation obtained in peptide CCN3 on incubation of cardiac troponin I with 3':5'-cyclic AMP-dependent protein kinase occurred at this residue. The data presented in Table 3 are derived from two independent determinations and suggest that the extent of phosphorylation of serine-20 before incubation with 3':5'-cyclic AMP-dependent protein kinase was approx. 0.3–0.6 mol of P/mol.

Although the major site of phosphorylation of cardiac troponin I with 3':5'-cyclic AMP-dependent protein kinase was serine-20, a significant degree of phosphorylation was also observed in peptide CCN2

(residues 49–150). A sample of ^{32}P-labelled peptide CCN2 obtained from a CNBr digest of ^{32}P-labelled troponin I was digested with trypsin. By gel filtration on Sephadex G-25 in 50 mM-NH$_4$HCO$_3$, followed by paper electrophoresis at pH 6.5, pH 2.0 and pH 3.5, six radioactive peptides were identified. One of these, peptide CCN2TA, contained 55 % of the total radioactivity recovered (about 40–45 % of that applied to the gel filtration column). The remaining 45 % of the recovered radioactivity was distributed between the other five peptides that were present in low yield. These could not be further characterized.

Peptide CCN2TA contained approx. 1 residue of homoserine per molecule (Table 2). As peptide CCN2TA was isolated from a characterized peptide obtained by CNBr digestion, the presence of homoserine indicated that it was derived from the C-terminal region of peptide CCN2. By comparing the amino acid analysis of the peptide with the published sequence of cardiac troponin I (Grand *et al.*, 1976) it was concluded that the sequence of peptide CCN2TA was Ile-Ser-Ala-Asp-Ala-Met. The phosphorylation site is therefore the serine residue at position 146 in rabbit cardiac-muscle troponin I. The specific radioactivity of peptide CCN2TA and the [γ-^{32}P]ATP with which the troponin I was phosphorylated were not significantly different. This indicates that serine-146 was not significantly phosphorylated before incubation of cardiac-muscle troponin I with 3':5'-cyclic AMP-dependent protein kinase.

Comparison of the rates of phosphorylation of two sites on cardiac-muscle troponin I

The location of the two sites of phosphorylation in different CNBr peptides of cardiac-muscle troponin I enabled a comparison to be made of their rates of phosphorylation. Since 97 % of the ^{32}P incorporated into peptide CCN3 after incubation of troponin I with [γ-^{32}P]ATP and 3':5'-cyclic AMP-dependent protein kinase was localized in serine-20 (see above) the rate of incorporation into the peptide is a close approximation of the rate of phosphorylation of serine-20. The rate of incorporation of ^{32}P into peptide CCN2, however, may not be such an accurate value for the rate of phosphorylation of serine-146. Only 55 % of the radioactivity incorporated into peptide CCN2 was present in peptide CCN2TA, which contains serine-146. We have no evidence as to whether the other radioactive peptides isolated from CCN2 are derived from serine-146 or from other sites of phosphorylation. Therefore the true rate of ^{32}P incorporation into serine-146 will be between 50 and 100 % of the rate of incorporation into peptide CCN2. Fig. 5 illustrates the time sequence of ^{32}P incorporation into the two CNBr peptides on incubation with 3':5'-cyclic AMP-dependent protein kinase. The results indicate that, even though serine-20
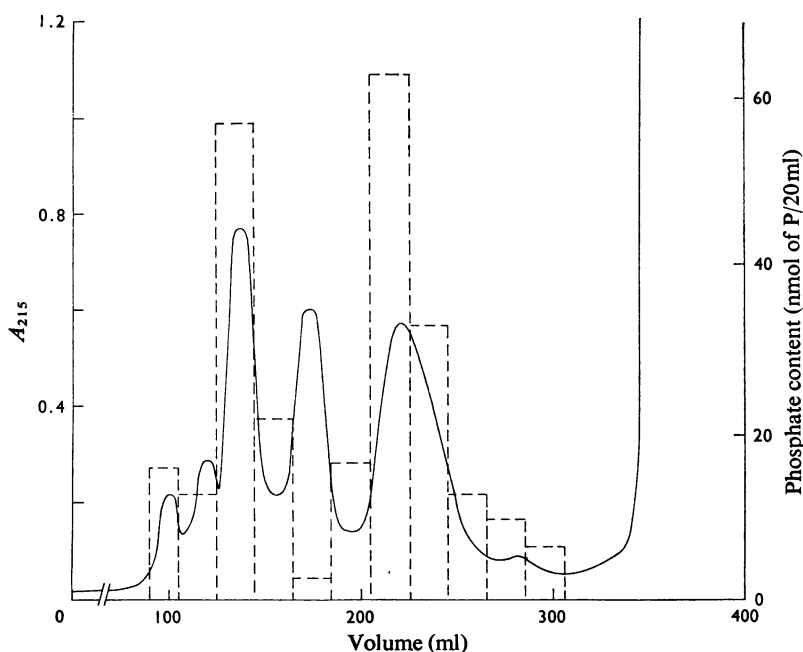
Fig. 4. *Distribution of phosphate in the CNBr peptides of cardiac troponin I*
Troponin I (5 mg, containing 290 μmol of phosphate) was digested with CNBr, and the digest carboxymethylated with iodoacetic acid (Moir *et al.*, 1974) and then fractionated by gel filtration on a column (2.5 cm × 110 cm) of Sephadex G-75 in 0.01 M-HCl. Fractions (5 ml) were collected and the A_{215} of each was determined. Every four consecutive fractions of the eluate were pooled, dried *in vacuo* and the total phosphate content of each determined. Dashed histograms represent total phosphate content (nmol) of each pooled fraction. ——, A_{215}.

Table 2. *Amino acid composition of the ^{32}P-containing tryptic peptides isolated from CNBr peptides CCN2 and CCN3 from rabbit cardiac troponin I*
Numbers in parentheses are the compositions for each peptide predicted from the sequence data of Grand *et al.* (1976). N.D., mobility of the peptide was not determined at this pH ; —, residue absent.

	Composition of peptide (mol/mol of peptide)		
	CCN2TA	CCN3TA	CCN3TB
Asp	1.2 (1)	0.9 (1)	1.0 (1)
Ser	1.1 (1)	1.2 (1)	1.2 (1)
Ala	1.8 (2)	0.8 (1)	0.9 (1)
Ile	0.8 (1)	— —	— —
Leu	0.3 —	— —	— —
Tyr	— —	0.7 (1)	0.7 (1)
Arg	— —	2.1 (2)	1.3 (1)
Hse	1.2 (1)	— —	— —
^{32}P content	0.95	0.26	0.20
$m_{6.5}$*	0.57	−0.10	0.10
$m_{2.0}$*	N.D.	0.85	0.48

* Electrophoretic mobility at the pH value indicated.

is partially phosphorylated before incubation with protein kinase, the initial rate of phosphorylation of this residue is much faster than that of serine-146.

Phosphorylation of the CNBr peptides or cardiac-muscle troponin I

The CNBr digest of cardiac-muscle troponin I could be phosphorylated at a significant rate by incubation with [γ-^{32}P]ATP and 3′:5′-cyclic AMP-dependent protein kinase. Direct quantitative comparisons with the rate of phosphorylation of the whole protein were not made, but the limited experiments carried out suggested that the whole protein was phosphorylated at 2–3 times the rate obtained with the digest. Fractionation of the digest after phosphorylation with [γ-^{32}P]ATP by gel filtration as illustrated in Fig. 6 indicated that radioactivity was present in peptides CCN1, CCN2 and CCN3. This suggested that the same sites were phosphorylated with the CNBr digest as the substrate as was the case with undigested troponin I.

Table 3. *Determination of the extent of phosphorylation of serine-20 in rabbit cardiac troponin I prepared by affinity chromatography*

Troponin I was incubated with $[\gamma\text{-}^{32}P]ATP$ and $3':5'$-cyclic AMP-dependent protein kinase, digested with CNBr and peptides were separated as described in Figs. 2 and 3. Specific radioactivities were calculated on the basis of the molar amounts determined from amino acid analysis. It has been assumed that all ^{32}P incorporated in peptide CCN3 is located at serine-20. Expts. 1 and 2 are two independent determinations.

|	Peptide CCN3TA		Peptide CCN3TB	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
(1) Amount of tryptic peptide containing 1 mmol of ^{32}P (mmol)	3.23	3.84	2.63	5.0
(2) Amount of non-radioactive phosphorylated tryptic peptide present in (1) (mmol)	2.23	2.84	1.63	4.0
(3) Amount of peptide CCN3 from which tryptic peptide isolated that contains 1 mmol of ^{32}P (mmol)	6.25	6.25	6.25	6.25
Extent of phosphorylation of serine-20 before incubation (%)	0.36	0.45	0.26	0.64
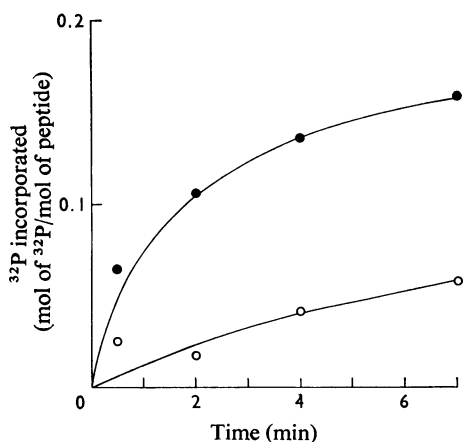
Fig. 5. *Comparison of the rates of phosphorylation of serine-20 and serine-146 in cardiac troponin I by bovine cardiac $3':5'$-cyclic AMP-dependent protein kinase*
Cardiac-muscle troponin I (3.0mg/ml) was incubated at 30°C with $3':5'$-cyclic AMP-dependent protein kinase (0.07mg/ml) and $[\gamma\text{-}^{32}P]ATP$ (1mM; $10\mu Ci/$ml). Samples (1.5ml) were removed at the times indicated, pipetted into an equal volume of ice-cold 15% (w/v) trichloroacetic acid, and washed five times with cold 7.5% (w/v) trichloroacetic acid. The ^{32}P-labelled troponin I samples were digested with CNBr in 0.1 M-HCl, and the digests carboxymethylated and fractionated by gel filtration on a column (2.5cm × 110cm) of Sephadex G-75 in 0.01 M-HCl as illustrated in Fig. 2. The amounts of peptides CCN2 and CCN3 were determined by amino acid analysis of the isolated fractions. ●, Incorporation of ^{32}P into peptide CCN3; ○, incorporation of ^{32}P into peptide CCN2.

Effect of troponin C on the phosphorylation of cardiac-muscle troponin I by $3':5'$-cyclic AMP-dependent protein kinase

Although both cardiac and fast-skeletal-muscle forms of troponin I form complexes with troponin C (either the cardiac or fast-skeletal forms) (Syska *et al.*, 1974; Head *et al.*, 1977), the phosphorylation of cardiac troponin I catalysed by $3':5'$-cyclic AMP-dependent protein kinase is not inhibited by troponin C (Cole & Perry, 1975). This is in contrast with skeletal troponin I (Perry & Cole, 1974), where inhibition is complete when the proteins are present in a 1:1 molar ratio. To investigate further the effect of troponin C on the phosphorylation of each site, cardiac troponin I was phosphorylated with $[\gamma\text{-}^{32}P]$-ATP and $3':5'$-cyclic AMP-dependent protein kinase in the absence and presence of an equimolar amount of skeletal troponin C. The ^{32}P-labelled troponin I was separated from the troponin C by chromatography on Sephadex G-75 in 0.01 M-HCl (see the Materials and Methods section), digested with CNBr and fractionated on Sephadex G-75 in 0.01 M-HCl. No significant differences were apparent in the amounts of ^{32}P present in each of the CNBr fragments when troponin I was phosphorylated for 25min with cyclic AMP-dependent protein kinase in the presence or absence of skeletal-muscle troponin C (Fig. 7). A similar pattern of phosphorylation was obtained if the time of phosphorylation was extended to 90min.

Discussion

The enzymic studies carried out on troponin I isolated from rabbit heart indicate that two sites of phosphorylation, serine-20 and serine-146, account
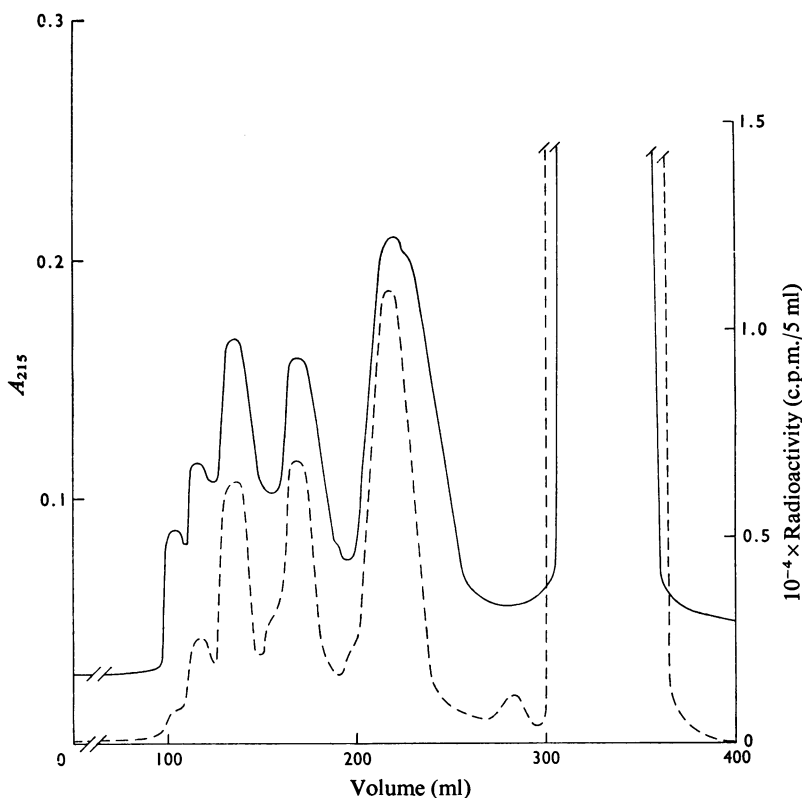
Fig. 6. *Phosphorylation of the CNBr peptides of cardiac troponin I by 3′:5′-cyclic AMP-dependent protein kinase*
A CNBr digest of rabbit cardiac-muscle troponin I (0.5 mg/ml) was incubated at 30°C with bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase (0.04 mg/ml) and [γ-^{32}P]ATP (1.0 mM; 10 μCi/ml) in a total volume of 5.0 ml under the conditions described in the Materials and Methods section. After 90 min, the pH was lowered to 2.0 by the addition of 1.0 M-HCl and the whole sample applied to a column (2.5 cm × 110 cm) of Sephadex G-75 equilibrated with 0.01 M-HCl. Fractions (5 ml) were collected and the A_{215} of every second fraction was determined. The large peak eluted between 300 and 350 ml is due to the presence of free [γ-^{32}P]ATP in the sample applied. ——, A_{215}; - - - -, radioactivity.

for virtually all the phosphate incorporated into the protein on incubation with 3′:5′-cyclic AMP-dependent protein kinase. Phosphorylation at serine-146 can be predicted, as it occupies an analogous position to serine-118 of fast-skeletal-muscle troponin I, which residue has previously been shown to be the main site of phosphorylation catalysed by this enzyme in skeletal troponin I (Moir *et al.*, 1974; Huang *et al.*, 1974). The site represented by serine-20, however, is of special interest in that it is located within the N-terminal region that is unique to cardiac troponin I (Grand *et al.*, 1976). This site was more rapidly phosphorylated than serine-146 even though it was partially phosphorylated before incubation with 3′:5′-cyclic AMP-dependent protein kinase. The phosphorylation of serine-20 must be largely responsible for the much more rapid rate of phosphorylation

of cardiac than of skeletal troponin I that is obtained with this protein kinase.

Partial phosphorylation at serine-20 in troponin I prepared by affinity chromatography accounted for only about 0.3–0.6 mol of P/mol, whereas the total covalently bound phosphate content of the protein was 1.0–1.5 mol of P/mol. It follows therefore that other site(s) were present that accounted for 0.7–0.9 mol of P/mol of cardiac-muscle troponin I. The studies on the distribution of phosphate among the CNBr peptides indicate that most of this phosphate must be located in the region represented by residues 1–48. In addition to serine-20 there are serine residues in positions 4, 32, 37 and 39, and threonine residues at position 26 and 46 (Grand *et al.*, 1976). Of these, serine-37 might be expected to be a site of phosphorylation catalysed by phosphorylase kinase, but
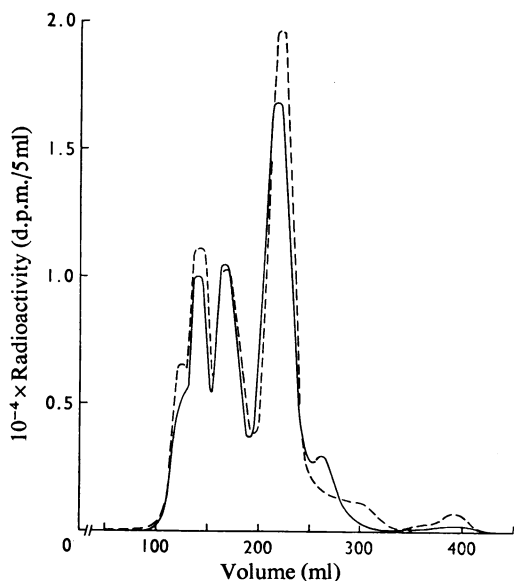
Fig. 7. *Gel filtration of CNBr digests of cardiac-muscle troponin I phosphorylated in the presence and absence of troponin C*

Samples of rabbit cardiac-muscle troponin I (12 mg) were phosphorylated with 3′:5′-cyclic AMP-dependent protein kinase (0.5 mg) and [γ-^{32}P]ATP in the presence or absence of rabbit skeletal-muscle troponin C (9 mg) for 25 min. The reactions, carried out in a total volume of 5.0 ml, were terminated by the addition of solid guanidine hydrochloride to a final concentration of approx. 6 M, and the ^{32}P-labelled troponin I samples isolated free of other components of the incubation by gel filtration as described in the Materials and Methods section. Both samples of troponin I were digested with CNBr. Equal amounts of each digest were carboxymethylated in 6 M-guanidine hydrochloride, 0.4 M-Tris (adjusted to pH 8.0 with 1.0 M-HCl)/1 mM-dithiothreitol/5 mM-iodoacetic acid and applied to columns (2.5 cm × 110 cm) of Sephadex G-75 equilibrated against and eluted with 0.01 M-HCl. Fractions (5 ml) were collected and the radioactivity was measured in every second fraction. ——, Incubation in the presence of troponin C; - - - -, incubation in the absence of troponin C.

not by 3′:5′-cyclic AMP-dependent protein kinase, by analogy with the site of phosphorylation specific for phosphorylase kinase in fast-skeletal-muscle troponin I. At the moment we have no direct enzymic evidence as to which of these sites are specific for phosphorylase kinase or which are phosphorylated *in vivo*, but it is unlikely that any are significant sites for phosphorylation by 3′:5′-cyclic AMP-dependent protein kinase.

The lack of inhibition by troponin C of the phosphorylation at the two sites identified is of relevance

to understanding the special features of the mode of interaction of troponin C with troponin I in cardiac muscle. The phosphorylation at serine-20 is not blocked in the presence of troponin C. It can therefore be concluded that the additional *N*-terminal peptide of cardiac troponin I is not involved in interaction with troponin C. Serine-146 occupies an analogous position to serine-118 in fast-skeletal-muscle troponin I. This site in skeletal-muscle troponin I is adjacent to the actin-binding site (Fig. 8) and its phosphorylation by 3′:5′-cyclic AMP-dependent protein kinase is markedly inhibited by troponin C (Perry & Cole, 1974). The fact that phosphorylation at serine-146 is not inhibited in cardiac-muscle troponin I allows the following conclusions to be made about the interaction that occurs *in vitro* between cardiac troponin I and troponin C. (1) Troponin C does not block sterically serine-146, the site close to the presumptive actin-binding site of cardiac troponin I. (2) Troponin C, although blocking the phosphorylase kinase-specific site(s) (Cole & Perry, 1975) and presumably binding in that region, does not impose a conformational change on cardiac troponin I that causes serine-20 and serine-146 to become unavailable to the 3′:5′-cyclic AMP-dependent protein kinase.

Studies on the sites phosphorylated in the perfused heart (Solaro *et al.*, 1976) correlate well with the results of the phosphorylation of the isolated proteins reported in the present paper. They enable the conclusion to be made that the decrease in the Ca^{2+}-sensitivity of the Mg^{2+}-stimulated actomyosin ATPase (adenosine triphosphatase) that occurs after incubation with 3′:5′-cyclic AMP-dependent protein kinase (Solaro *et al.*, 1976; H. A. Cole & S. V. Perry, unpublished work) may be directly related to the phosphorylation of serine-20. Ray & England (1976) have reported an increase in the Ca^{2+}-sensitivity of the Mg^{2+}-stimulated ATPase after dephosphorylation, but no information is available from their studies as to which sites are affected by the phosphatase.

Several workers (Bylund & Krebs, 1975; Daile *et al.*, 1975; Kemp *et al.*, 1975; Williams, 1976) have discussed the specificity of 3′:5′-cyclic AMP-dependent protein kinase, and it has been suggested that the enzyme recognizes a potential phosphorylation site by the presence of a basic residue near to the serine. In particular, Yeaman *et al.* (1976) have concluded that phosphorylation is directed to a serine residue by the presence of at least two adjacent basic amino acids on the *N*-terminal side of the serine residue and separated from the serine by either one or two intermediate residues. Neither serine-20 or serine-146 (Fig. 1) in cardiac-muscle troponin I strictly satisfies the criteria suggested by Yeaman *et al.* (1976), although phosphorylation is more rapid at that site the sequence of which is more similar to the proposed recognition sequence. Comparison of the rates of phosphorylation of the different sites in
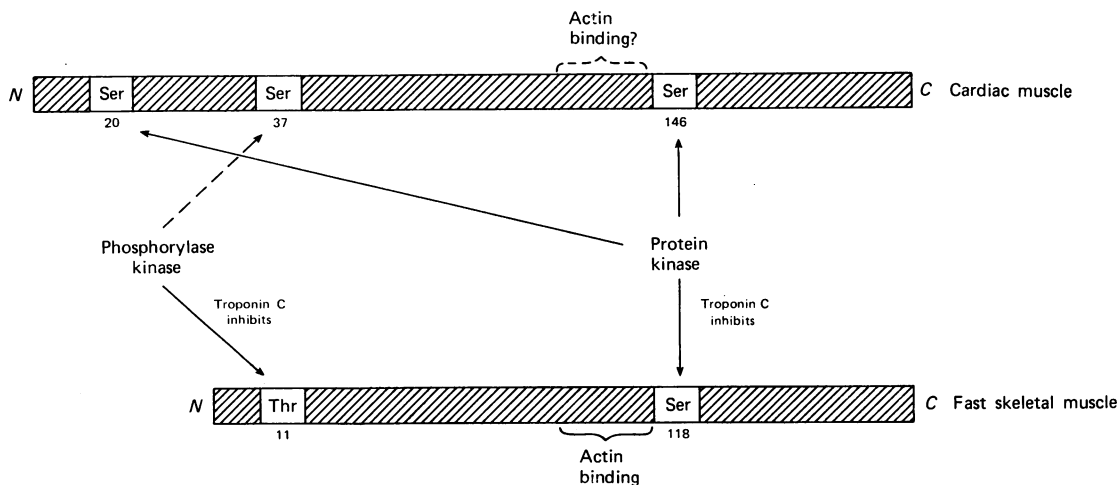
Fig. 8. *Phosphorylation sites of cardiac- and fast-skeletal-muscle troponin I*
Experimental evidence suggests that troponin C can interact with the N-terminal region and the actin-binding region of fast-skeletal-muscle troponin I. It is presumed that the region of the sequence of cardiac-muscle troponin I analogous to that of fast-skeletal-muscle troponin I, which possesses inhibitory activity on the Mg^{2+}-stimulated actomyosin ATPase, is the actin-binding sequence (incorporating data from: Perry & Cole, 1974; Moir *et al.*, 1974; Cole & Perry, 1975; Wilkinson & Grand, 1975; Syska *et al.*, 1976; Grand *et al.*, 1976). N, N-Terminus; C, C-terminus.

skeletal-muscle and cardiac troponin I with those obtained with other well-defined substrates should help to extend our understanding of the factors determining the specificity of this widely distributed enzyme.

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