THE PHOSPHORYLATION SITES OF TROPONIN I FROM WHITE SKELETAL MUSCLE OF THE RABBIT

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1. Introduction

Since the original demonstration of the phosphorylation of troponin-I by a 3′:5′-cyclic AMP-dependent protein kinase [1], there have been a number of somewhat contradictory reports [2–4] as to which components of the complex can be phosphorylated by phosphorylase kinase and a 3′:5′-cyclic AMP-dependent protein kinase. From recent studies in this laboratory [5] we conclude that there are phosphorylation sites on both troponin-I and troponin-T and that the troponin complex isolated from white skeletal muscle of the rabbit contains one molecule of phosphate covalently bound to troponin-T. Prolonged incubation of either troponin-T or the troponin complex with phosphorylase kinase increases the phosphate content of troponin-T up to about three moles per mole. Troponin-I incorporates up to 1.6 moles of phosphorus when incubated with phosphorylase kinase but this phosphorylation is markedly inhibited in the presence of troponin-C [5–7]. This effect is due to the strong complex formed between troponin-I and troponin-C [8,9] and is responsible for the different patterns of phosphorylation obtained with troponin [3,4] and with troponin-B [2].

In this communication we report the amino acid sequences around the two residues of troponin-I that are phosphorylated by phosphorylase kinase from rabbit skeletal muscle and a 3′:5′-cyclic AMP-dependent protein kinase from bovine heart. Essentially similar findings are reported in the accompanying communication by Huang et al. [14].

2. Materials

Troponin was purified from rabbit skeletal muscle by the method of Ebashi et al. [10], and separated into its components by chromatography on DEAE-cellulose [5]. Phosphorylase *b* kinase was isolated from rabbit muscle by the procedure of Cohen [11]. 3′:5′-cyclic AMP-dependent protein kinase, prepared from beef heart, was obtained from Sigma (London) Chemical Co. Ltd., Kingston-on-Thames, Surrey, UK. The cyanogen bromide fragments, CN5 and CN10 from unphosphorylated troponin-I were isolated as described previously [12]. The nomenclature of the cyanogen bromide fragments is that used previously [12], the suffix P indicating that the fragments were phosphorylated.

3. Methods

Phosphorylation with phosphorylase kinase was carried out by the procedure of Perry and Cole [5], except that theophylline was omitted from the incubation mixture. For phosphorylation with the bovine cardiac protein kinase, troponin-I (2 mg/ml) was incubated at 30°C for 6 hr with the enzyme (0.05 mg/ml) in 10 mM sodium glycerophosphate (pH 6.8), containing 20 mM NaF, 10 mM magnesium acetate, 1.5 mM EGTA, 0.05 mM 3′:5′-cyclic AMP and 1 mM [γ-^{32}P] ATP. Total phosphate determinations on troponin-I and determination of ^{32}P were carried out as described previously [4].

Phosphorylated peptides were isolated in the following manner. ^{32}P-labelled troponin-I was dissolved in 0.1 N HCl (20 mg/ml) and incubated with a 100-fold molar excess of cyanogen bromide for 40 hr at 23°C. Cysteine residues were then carboxymethylated with iodoacetic acid [12]. The cyanogen bromide fragments were fractionated by chromatography on a column (2.5 × 110 cm) of Sephadex G-50 in 6 M urea, 0.2 M sodium formate, pH 3.5. Fractions were desalted on a column (2.0 × 100 cm) of Sephadex G-10 in 0.01 N HCl.

Fractions containing radioactivity were further purified by high voltage electrophoresis and ^{32}P-labelled peptides located by autoradiography. Enzymic digests of protein and cyanogen bromide fragments were carried out in 1% NH_4HCO_3 pH 7.9 at 37°C for 3 hr using an enzyme/substrate ratio of 1:50. Amino acid analysis and amino acid sequence determinations were performed as described previously [12].

4. Results

4.1. *Incorporation of ^{32}P into troponin-I by phosphorylase b kinase*

Preparations of troponin-I contained 0.06 mole of phosphorus per mole. After incubation with phosphorylase kinase, the phosphorus content was increased to between 0.90 and 1.25 moles per mole.

Gel chromatography of a cyanogen bromide digest of ^{32}P-labelled troponin-I indicated that covalently bound phosphorus had been incorporated mainly into three fragments (fig. 1, fractions A, B and C). On examination by paper electrophoresis, fractions B and C were each found to contain one major radioactive component. The amino acid compositions of these phosphorylated peptides are given in table 1. CN5-P (from peak B) contained 0.79 mole ^{32}P/mole and CN10-P (from peak C) contained 0.83 mole ^{32}P/mole. As is shown by the distribution of ^{32}P between peaks B and C (fig. 1), the yield of CN5-P was considerably greater than that of CN10-P; however, the non-phosphorylated form of CN10 was also isolated from the digest, the ratio of phosphorylated to non-phosphorylated peptide being about 35:65.

Tryptic digestion of CN5-P gave rise to a single radioactive peptide CN5-T2. This contained 0.91

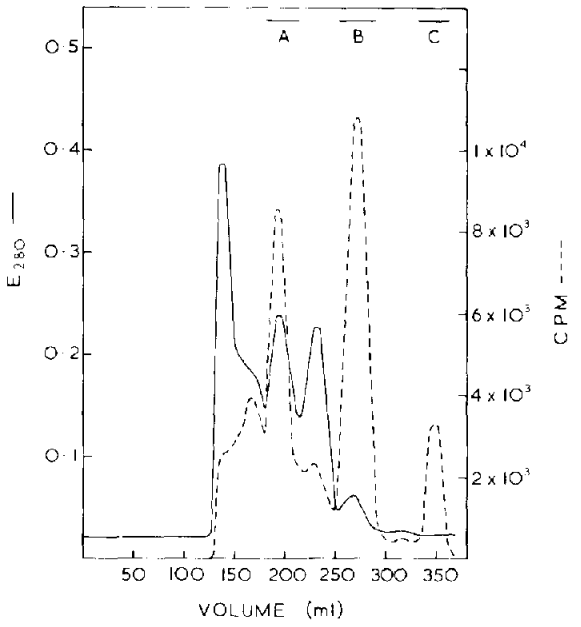


Fig. 1. Chromatography on Sephadex G50 of the cyanogen bromide peptides derived from ^{32}P-labelled troponin-I. Troponin-I labelled by incubation with phosphorylase kinase in the presence of [γ-^{32}P]ATP as described by Perry and Cole [5]. 100 mg of digest were applied to a column (2.5 × 110 cm) that had previously been equilibrated against 6M urea, 0.2 M sodium formate (pH 3.5), 5 ml fractions were collected and appropriate tubes pooled into fractions A, B or C as indicated by the horizontal bars.

mole ^{32}P/mole and its amino acid composition (table 1) indicated that the single threonine residue in CN5-P was phosphorylated. Fraction A was also digested with trypsin and the only radioactive peptide obtained was identical with CN5-P-T2. Thus the large phosphorylated fragment in peak A did not contain a third phosphorylation site but was a partial degradation product resulting from incomplete cleavage during the cyanogen bromide digest.

4.2. *Incorporation of ^{32}P into troponin-I by protein kinase*

Less than one mole of phosphorus was incorporated into troponin-I on prolonged incubation with the bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase (6 hr). Tryptic digestion of ^{32}P-labelled troponin-I gave rise to two major radioactive peptides, Ta and Tb (see table 1). Both peptides come from the same part of troponin-I as CN10 and their com-

Table 1
Amino acid composition of phosphorylated peptides from troponin-I

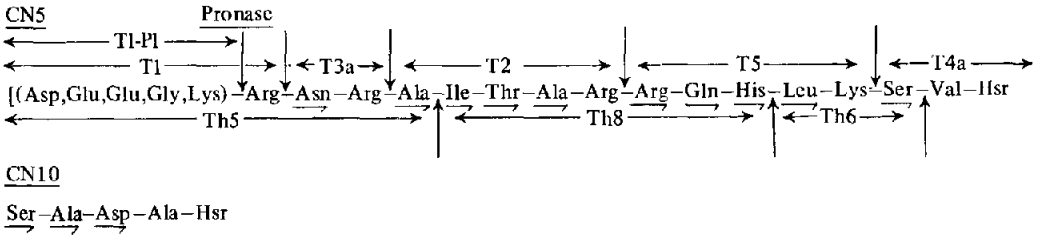
Enzyme	Phosphorylase *b* kinase				Protein kinase	
	CN5-P	CN5-P-T2	CN10-P	CN10	Ta	Tb
Asp	2.25	(0.20)	1.04	1.04	1.39	1.40
Thr	0.75	0.91	–	–	–	–
Ser	1.07	(0.29)	0.67	0.65	1.09	1.02
Glu	3.32	(0.20)	(0.41)	(0.28)	(0.21)	(0.43)
Gly	1.24	(0.25)	(0.46)	(0.34)	(0.41)	(0.32)
Ala	1.90	1.91	2.02	2.08	2.03	2.12
Val	1.10	–	–	–	0.61	–
Ile	0.99	1.08	–	–	–	–
Leu	1.12	–	–	–	1.20	1.05
His	1.00	–	–	–	–	–
Lys	1.63	–	–	–	0.81	0.95
Arg	3.90	1.10	–	–	0.79	–
Hsr	1.00	–	0.96	1.17	–	–
Met	–	–	–	–	1.50	1.60
moles ^{32}P/mole	0.79	0.91	0.83	–	0.54	0.74

Values expressed as mole amino acid/mole peptide. Values in brackets indicate impurities.

bined sequence is: Val–Arg–Met–Ser P–Ala–Asp–Ala–Met–Leu–Lys (J.M. Wilkinson, unpublished work). The unphosphorylated versions of Ta and Tb were not found, but their presence cannot be ruled out. Ta and Tb together accounted for 90% of the ^{32}P incorporated into troponin-I by protein kinase and thus if the threonine residue in CN5 was phosphorylated it could only be to the extent of 10% or less.

4.3. *Amino acid sequence of CN5 and CN10*

The sequences were determined using material from unphosphorylated troponin-I. Peptides were isolated by electrophoresis from tryptic and thermolysin digests of CN5 and sequence information obtained by the dansyl–Edman procedure. Peptide T1 possessed no free amino group and must therefore be the N-terminal peptide of troponin-I; its mobility at pH 6.5 was consistent with it being N-acetylated but this has yet to be confirmed. The sequence of CN10 was obtained directly by the dansyl–Edman procedure. The two sequences and a summary of the evidence by which they were obtained are shown in fig. 2.



The square bracket indicates a blocked N-terminal group. T and Th indicate tryptic and thermolysin peptides respectively. ⟶ Represents one step of the dansyl–Edman procedure.

Fig. 2. Amino acid sequence of the cyanogen bromide fragments CN5 and CN10 from troponin-I.

5. Discussion

The site in the troponin molecule most readily phosphorylated by phosphorylase kinase from rabbit skeletal muscle is the threonine residue in position 11. Whereas phosphorylation of this threonine residue was virtually complete under the conditions employed, the only other phosphorylated site detected in troponin-I, a serine residue in position 118 (J. M. Wilkinson, unpublished observations) was never more than 35% phosphorylated. The variation in the extent of phosphorylation of troponin-I by phosphorylase kinase observed in these and earlier experiments [5] probably reflects differences in the extent of phosphorylation at serine 118. In contrast, phosphorylation of troponin-I by the bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase was largely restricted to serine 118, 90% of the total phosphate being incorporated in this site. Thus although the cardiac enzyme catalyses phosphorylation principally at serine 118 of troponin-I from rabbit white skeletal muscle the 3′:5′-cyclic AMP-dependent protein kinase from the latter muscle appears to catalyse the phosphorylation of another serine in addition (see preceding article, this issue pp. 249–252). The observation [5] that the phosphorylation of troponin-I by phosphorylase kinase in the presence of troponin-C was more strongly inhibited than in the case with the bovine cardiac protein kinase is consistent with our findings that the major sites of phosphorylation by these two enzymes are different.

The amino acid sequence of the major phosphorylated region of troponin-I, Ala–Ile–Thr–Ala–Arg, shows a relatively high degree of similarity to the amino acid sequence around the serine phosphate of phosphorylase *a*, Gln–Ile–Ser–Val–Arg [13]. There is, however, little similarity between these sequences and the second phosphorylation site on troponin-I and those two of the three phosphorylated regions of troponin-T that have been determined (A.J.G. Moir and S.V. Perry, unpublished observations). Although all five of these sites are phosphorylated by phosphorylase kinase preparations, the rates are low compared to those obtained with phosphorylase *b* as substrate. This suggests that the specificity of phosphorylase kinase is much broader than was previously supposed. At this stage it cannot be excluded, however that the phosphorylase kinase preparations contain traces of other, as yet uncharacterised, kinases of different specificity and which may be responsible for phosphorylation of some of the sites on troponin.

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References

[1] Bailey, C. and Villar-Palasi, C. (1971) Federation Proc. Fed. Amer. Soc. Exp. Biol. 30, 1147.
[2] Stull, J.T., Brostrom, C.O. and Krebs, E.G. (1972) J. Biol. Chem. 247, 5272–5274.
[3] Pratje, E. and Heilmeyer, L.M.G. (1972) FEBS Letters 27, 89–93.
[4] Perry, S.V. and Cole, H.A. (1973) Biochem. J. 131, 425–428.
[5] Perry, S.V. and Cole, H.A. (1974) Biochem. J. In the press.
[6] Perry, S.V. and Cole, H.A. (1974) Biochem. Soc. Trans. 2, 89–90.
[7] Krebs, E.G. Personal Communication.
[8] Perry, S.V., Cole, H.A., Head, J.F. and Wilson, F.J. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 251–262.
[9] Head, J.F. and Perry, S.V. (1974) Biochem. J. 137, 145–154.
[10] Ebashi, S., Wakabayashi, T. and Ebashi, F. (1971) J. Biochem. 69, 441–445.
[11] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[12] Wilkinson, J.M. (1974) FEBS Letters 41, 166–168.
[13] Nolan, C., Novoa, W.B., Krebs, E.G. and Fischer, E.H. (1964) Biochemistry 3, 542–551.