THE AMINO ACID SEQUENCES OF THE PHOSPHORYLATED SITES IN TROPONIN-I FROM RABBIT SKELETAL MUSCLE

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1. Introduction

Troponin functions in muscle contraction as a regulatory protein which sensitizes the interaction of actin and myosin to calcium [1]. This protein is composed of three distinct subunits [2]: a calcium binding protein (TN-C), a tropomyosin binding protein (TN-T), and an inhibitory protein (TN-I).

Bailey and Villar-Palasi [3] first reported the phosphorylation of TN-I by cyclic AMP-dependent protein kinase from rabbit skeletal muscle. It was later shown that phosphorylase kinase also catalyzes the phosphorylation of TN-I [4], and that phosphorylase phosphatase catalyzes the dephosphorylation of the phosphorylated product [5]. TN-I phosphorylated by protein kinase was also dephosphorylated by phosphorylase phosphatase [6]. Subsequently TN-T was identified as an additional component of the troponin complex which was phosphorylated by protein kinase [7–9] and phosphorylase kinase [8,9], and dephosphorylated by phosphorylase phosphatase [9]. The physiological significance of these reactions is unknown at the present time.

Protein kinase recognizes a number of proteins as substrates [10] whereas phosphorylase kinase [11] and phosphorylase phosphatase [12] have been characterized as being relatively specific for phosphorylase as a substrate. The purpose of this communication is to examine the amino acid sequences of the phosphorylated sites in TN-I and compare them to the phosphorylated site in phosphorylase *a*. This comparison may indicate if there are any unique requirements in the primary structure that may be important for determining the substrate specificity of these enzymes. A similar result is reported in the accompanying communication by A. J. G. Moir, J. M. Wilkinson and S. V. Perry (see following article, this issue, pp. 253–256).

2. Materials and methods

Troponin was isolated from rabbit skeletal muscle [13] and separated into its individual subunits [2,14]. The endogenous protein-bound phosphate in TN-I was 0.02 moles/mole TN-I. Phosphorylase kinase [15] and the catalytic subunit of the cyclic AMP-dependent protein kinase [16] were also prepared from rabbit skeletal muscle. For the phosphorylation by phosphorylase kinase, TN-I (1.1 mg/ml) was incubated with 30 μg/ml enzyme for 2.5 hr at 30°C in the presence of 20 mM N-Tris [hydroxymethyl] methyl-2 aminoethane sulfonic acid (pH 7.8), 50 mM magnesium acetate, 15 mM β-mercaptoethanol, 0.01 mM $CaCl_2$ and 1 mM [γ-^{32}P]ATP (56 cpm/pmole). A maximum incorporation of 1 mole ^{32}P/mole TN-I was achieved in 90 min. For the phosphorylation by protein kinase, TN-I (1.0 mg/ml) was incubated with 4 μg/ml enzyme at 30°C for 6 hr in a reaction mixture containing 0.5 mM [γ-^{32}P]ATP (25 cpm/pmole), 50 mM 2[*N*-morpholino]ethane sulfonic acid (pH 6.5), 10 mM magnesium acetate and 15 mM β-mercaptoethanol. This resulted in 1.9 moles ^{32}P incorporated/mole TN-I. Cyanogen bromide (BrCN) digestion [17,18], maleylation [19] and S-carboxymethylation [20] were performed by published procedures. Enzymic digestions with trypsin, chymotrypsin and thermolysin were routinely carried out

in 0.1 M NH_4HCO_3 at 40°C using 1:100 ratio of enzyme to substrate for 4 hr. Gel filtration of peptides using Sephadex G-25 and G-50 was performed in 30% acetic acid. Ion exchange chromatography using SP-Sephadex C-25 [21] or high voltage electrophoresis at pH 1.9 on Whatman No. 3 MM paper were used to purify the various peptides. Amino acid sequence studies were performed using the manual Edman method [22] and carboxypeptidases. The assignment of amide or acidic residues was based on hydrolysis by aminopeptidase M or carboxypeptidase A.

3. Results

3.1. *Sites phosphorylated by phosphorylase kinase*

To determine which amino acid residues were phosphorylated by phosphorylase kinase, 100 mg of phosphorylated TN-I were digested with BrCN. The resulting peptides were fractionated on Sephadex G-25 and G-50 columns, and then purified by high voltage electrophoresis. One major phosphopeptide, BrCN-3 (representing about 70% of the ^{32}P incorporated), along with two minor phosphopeptides, BrCN-1 and BrCN-2, were isolated and their amino acid sequences determined (fig. 1). Four major peptides were isolated from a thermolysin hydrolysis of peptide BrCN-3. The ordering of Th-1 and Th-2 peptides was based on an overlapping tryptic peptide with a sequence of Ala–Ile–Thr(P)–Ala–Arg, which has been reported previously [6].

Determinations of the NH_2-terminal residue by the Edman method of TN-I and peptides BrCN-3, Th-1, Th-1-a and Th-1-T-1 were unsuccessful, indicating that the NH_2-terminals of the peptides and TN-I were blocked. This suggests that BrCN-3 may be derived from the NH_2-terminal segment of TN-I molecule. Treatment of peptide Th-1-T-1 with acid under different conditions [23,24] followed by Edman degradation showed glycine to be the NH_2-terminal residue. The nature of the blocking group at present is unknown, but presumably it is a N-acetyl group.

3.2. *Sites phosphorylated by protein kinase*

The sites in TN-I phosphorylated by protein kinase were determined in a similar way. Following reduction, carboxymethylation, maleylation, and hydrolysis of the phosphorylated protein by trypsin, one phosphorylated peak was observed on Sephadex G-25 and G-50 columns. After demaleylation and chromatography on SP-Sephadex C-25, the phosphopeptides were redigested with trypsin. The resulting four phosphopeptides were purified by gel filtration and high voltage electrophoresis, and then sequenced (fig. 2). There was no evidence of a tryptic phosphopeptide Ala–Ile–Thr(P)–Ala–Arg corresponding to the major site phosphorylated by phosphorylase kinase.

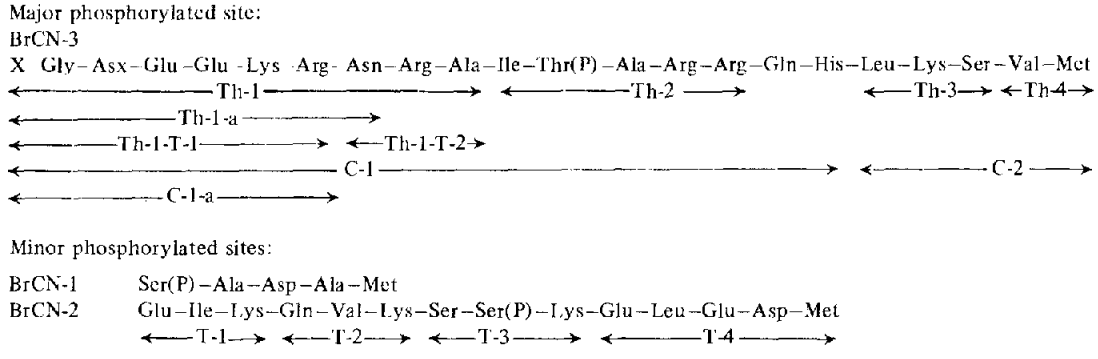

Fig. 1. Phosphorylated sites of TN-I incubated with phosphorylase kinase. Th = peptides derived from thermolysin hydrolysis; T = peptides derived from tryptic cleavage; C = peptides derived from chymotrypsin digestion. The ordering of T-1 and T-2 is based on the Edman degradation of the first 5 residues of the intact BrCN-2 peptide. A personal communication from A. J. G. Moir, J. M. Wilkinson and S. V. Perry indicated the presence of Gln–His between Th-2 and Th-3 peptides. The sequence was then confirmed by carboxypeptidase a hydrolysis of C-1 peptide.

Tm-T-1-C	Ser(P)–Val–Met–(Glx, Leu)
Tm-T-2-Th	Ser(P)–Val–Met–(Gln, Leu)
Tm-T-3	Val–Arg–Met–Ser(P)–Ala–Asx–(Ala,Met)–Leu–Lys
Tm-T-4	Met–Ser(P)–Ala–Asx–(Ala,Met)–Leu–Lys

Fig. 2. Sites in TN-I phosphorylated by protein kinase. Four phosphopeptides were isolated following a tryptic (Tm) digestion of maleylated TN-I, and a redigestion with trypsin (T) after demaleylation (see Materials and methods). One phosphopeptide was then digested with chymotrypsin (C) and another with thermolysin (Th).

4. Discussion

When a comparison is made of an extended portion of the phosphorylated region of phosphorylase *a*, which has the sequence of *N*–Ac–Ser–Arg–Pro–Leu–Ser–Asp–Gln–Glu–Lys–Gln–Ile–Ser(P)–Val–Gly–Leu–Ala–Ala–Val–Glu–Asn–Val–Thr–Glu–Leu–Lys–Lys (E. Fischer, personal communication), with the sequence of the TN-I region (fig. 1) phosphorylated by phosphorylase kinase, no appreciable homology is evident. However, if a comparison is made in the immediate vicinity of the phosphorylated sites, which have the sequences of Lys–Gln–Ile–Ser(P)–Val–Arg for phosphorylase *a*, and Arg–Ala–Ile–Thr(P)–Ala–Arg for TN-I, definite similarities are apparent. Two residues are identical (Ile and COOH-terminal Arg) and three others represent conservative changes (Arg for Lys, Thr for Ser, Ala for Val). The similarities suggest that a certain segment of the primary sequence may play an important role in the recognition of a substrate by phosphorylase kinase. The two minor phosphorylated sites, BrCN-1 and BrCN-2, show no resemblance to the phosphorylase *a* site.

Protein kinase incorporates two moles ^{32}P/mole TN-I and the primary structures of the isolated phosphorpeptides suggest that only two sites are phosphorylated. Although one of these sites (Tm-T-1-C and Tm-T-2-Th) shows no resemblance to any of the sites phosphorylated by phosphorylase kinase, the other site (Tm-T-3 and Tm-T-4) is identical to one of the minor sites (BrCN-1) observed in the phosphorylation by phosphorylase kinase. The BrCN-1 site could be due to a contamination of phosphorylase kinase with protein kinase. However, kinetic analyses of the phosphorylation reaction with phosphorylase kinase suggest the contamination would not cause significant TN-I phosphorylation (Stull, unpublished observations). The lack of similarity between the two sites phosphorylated by protein kinase indicates either that the primary structural determinants are not very stringent for determining substrate specificity or that higher orders of structure are more important.

The fact that phosphorylase phosphatase removes phosphate incorporated into TN-I by both phosphorylase kinase and protein kinase at a rate greater than the dephosphorylation of phosphorylase *a* (P. J. England, unpublished observations) indicates the primary structure of the phosphorylated site is not an important determinant of specificity for this phosphatase.

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