Phosphorylase kinase phosphorylation of skeletal-muscle troponin T

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Rabbit skeletal-muscle troponin T was phosphorylated by a standard preparation of phosphorylase kinase [Cohen (1973) *Eur. J. Biochem.* **34**, 1–14] and by fractions obtained after chromatography of phosphorylase kinase on phosphocellulose. The original preparation of phosphorylase kinase phosphorylated at least two sites, one of which was serine-1. The second and probably the third sites were presumably located in the peptide flanked by amino-acid residues 147 and 161 of troponin T. Fractions of phosphorylase kinase was adsorbed on phosphocellulose phosphorylated only the second site. Tightly adsorbed fractions possessed high troponin T kinase and phosvitin kinase activities and phosphorylated only serine-1 of troponin T. The results suggest that standard preparations of phosphorylase kinase are contaminated by troponin T kinase, which can phosphorylate serine-1 of troponin T.

The last decade has been marked by an increased interest in the investigation of contractile and regulatory protein phosphorylation (England *et al.*, 1973; Perry, 1979). Phosphorylase kinase (EC 2.7.1.38) can play an important role in troponin phosphorylation. Phosphorylase kinase phosphorylates threonine-11 in skeletal-muscle troponin I (Huang *et al.*, 1974; Moir *et al.*, 1974) and serine-72 in cardiac troponin I (Moir *et al.*, 1977). In both cases the primary sequence of the phosphorylated sites is similar to the phosphorylation site of phosphorylase *b*. Phosphorylase kinase incorporates 3 mol of phosphate/mol of skeletal-muscle troponin T (Moir *et al.*, 1977). The sequence of only one of these sites (around serine-149–serine-150) is similar to the sequence in phosphorylase *b*. The high specificity of phosphorylase kinase is well documented (Cohen, 1974), so two hypotheses were proposed to explain troponin T phosphorylation. According to the first hypothesis (Dickneite *et al.*, 1978), phosphorylase kinase contains two catalytic centres, one of which catalyses phosphorylase *b* phosphorylation, and the second troponin T phosphorylation. According to the second hypothesis (Moir *et al.*, 1977; Perry, 1979) even highly purified preparations of phosphorylase kinase are contaminated by protein kinase(s), capable of phosphorylating troponin T. The present paper describes the separation of phosphorylase kinase and troponin T kinase activities of a standard phosphorylase kinase preparation (Cohen, 1973) by using ion-exchange chromatography.

Materials and methods

The isolation of the whole troponin complex and troponin T from rabbit skeletal muscle and the dephosphorylation of troponin T by *Escherichia coli* alkaline phosphatase have been described previously (Staprans *et al.*, 1972; Wilkinson, 1974; Gusev *et al.*, 1978). Phosvitin was isolated by the method of Joubert & Cook (1958). Rabbit skeletal-muscle phosphorylase *b* was prepared by the method of Fisher & Krebs (1962), recrystallized three times and freed from nucleotides by Norit A treatment. The specific activity of the enzyme was 40 units/mg, as determined by the method of Illingworth & Cori (1953). Phosphorylase kinase was isolated by the method of Cohen (1973) and was further purified on phosphocellulose. The incubation mixture of the following composition was used for the protein kinase activity determination: 10 mM-sodium glycerol-3-phosphate, 10 mM-Tris (pH 8.0), 10 mM-Magnesium acetate, 0.5 mM-CaCl₂, 0.1 mM-ATP, which contained [γ -³²P]ATP (5×10^5 – 2×10^6 c.p.m./100 μ l). The concentrations of troponin T and phosvitin varied from 0.4 to 1.0 mg/ml, that of phosphorylase *b* between 7 and 10 mg/ml. The protein kinase activity was determined by the method of Reimann *et al.* (1971), after pipetting 40 μ l of incubation mixture on to a Whatman 3MM filter paper. The original preparations of phosphorylase kinase isolated by the method of Cohen (1973) have the specific activity 0.7–1.0 μ mol/mg per min under the conditions described. ³²P-labelled

troponin T was obtained after incubation of dephosphorylated troponin T with different protein kinase fractions. ^{32}P -labelled troponin T was separated from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ on a column of Bio-Gel P-10 in 0.1 M-HCl, freeze-dried and hydrolysed by trypsin (Serva; 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one-treated) in 0.1 M-Tris/HCl (pH 8.0). The ratio by weight of trypsin/troponin T was 3.3/100. The troponin T tryptic hydrolysate was subjected to chromatography on a Bio-Gel P-4 column (1.2 cm $\times$ 100 cm) in 0.1 M-HCl; the A_{206} was recorded continuously. The radioactivity of fractions was measured by the Čerenkov method. The protein concentration was determined by the method of Spector (1978) with bovine serum albumin as standard. The homogeneity of protein fractions was assayed by gel electrophoresis in the presence of sodium dodecyl sulphate (Laemmli, 1970).

Results

The rate of troponin phosphorylation by original preparations of phosphorylase kinase was usually 50–500 times less than that of phosphorylase phosphorylation. These data are in agreement with previous publications (Moir *et al.*, 1977; Dickneite *et al.*, 1978). The troponin T/phosphorylase *b* phosphorylation ratio varied for different phos-

phorylase kinase preparations. We supposed that phosphorylase kinase preparations were contaminated by troponin T kinase (Gusev *et al.*, 1978; Kumon & Villar-Palasi, 1979). This enzyme is tightly adsorbed on phosphocellulose (Dobrovolskii, 1979) and it seemed promising to use phosphocellulose chromatography for separation of troponin T kinase from phosphorylase kinase.

The original preparations of phosphorylase kinase were weakly adsorbed on phosphocellulose and the largest part of the protein was either not absorbed or was eluted by 50 mM-potassium phosphate (see Fig. 1). The fractions unabsorbed on phosphocellulose possessed a high phosphorylase kinase activity, although a very low phosphorylase kinase activity was detected throughout the whole elution profile. The specific activity and protein composition of the fractions unabsorbed on phosphocellulose were similar to the corresponding parameters of the original phosphorylase kinase. These fractions were capable of phosphorylating troponin T, and the ratio of phosphorylase *b* phosphorylation to that of troponin T phosphorylation was usually higher than 100.

A high troponin T kinase activity was detected in the fractions tightly adsorbed on phosphocellulose, and eluted by 0.35–0.4 M-potassium phosphate buffer (Fig. 1, fractions 49–52). In most cases the

Fig. 1. Chromatography of the original preparation of phosphorylase kinase (Cohen, 1973) on phosphocellulose. An original preparation of phosphorylase kinase, obtained after gel filtration on Sepharose 4B was applied to a phosphocellulose column (1.2 cm $\times$ 12 cm) equilibrated with 50 mM-potassium phosphate (pH 7.0), containing 2 mM-EDTA and 2 mM-mercaptoethanol (buffer A). The column was washed with buffer A (fractions 8–11) and the column was eluted with a linear gradient (total volume 190 ml), consisting of equal volumes of 50 mM- and 500 mM-potassium phosphate buffer (fractions 12–55). The assay mixture for the determination of protein kinase activity contained 1 μl of eluate (when phosphorylase *b* was used as the substrate) or 10 μl of eluate (when troponin T was used as the substrate). The composition and the volume of assay mixture are given in the Materials and methods section. The incubation was carried out for 10 min at 30°C.

phosphorylase kinase activity of these fractions was only three to five times that of troponin T kinase and sometimes, when small amounts of phosphorylase kinase were subjected to the chromatography, only the troponin T kinase activity was detected in these fractions. By using phosvitin as substrate we have found that both original preparations of phosphorylase kinase and fractions tightly bound to phosphocellulose possess the phosvitin kinase activity. It is known that troponin T kinase phosphorylates phosvitin (Kumon & Villar-Palasi, 1979; Dobrovolskii, 1979; Gusev *et al.*, 1980), so the detection of troponin T and coincident phosvitin kinase activity in the fractions tightly bound to phosphocellulose suggests that standard preparations of phosphorylase kinase (Cohen, 1973) are contaminated by troponin T kinase.

Troponin T kinase phosphorylates only serine-1, located in the largest (40 amino acids) tryptic peptide of troponin T (Pearlstone *et al.*, 1976; Gusev *et al.*, 1978). This peptide is quantitatively separated from all other small peptides and is eluted in the

homogeneous state during gel filtration of the troponin T tryptic hydrolysate (Moir *et al.*, 1977). So the distribution of ^{32}P among the tryptic peptides of troponin T, phosphorylated by original phosphorylase kinase, and different fractions obtained after phosphocellulose can therefore be indicative of the possible contamination of the original phosphorylase kinase preparation by troponin T kinase.

The original preparations of phosphorylase kinase phosphorylated at least two sites in troponin T (Fig. 2a). One of them was located in the largest *N*-terminal peptide and is presumably serine-1 (Moir *et al.*, 1977; Gusev *et al.*, 1978). The other (or two other) site of phosphorylation was located in a small peptide and is presumably represented by serine-149–serine-150 and/or serine-156–serine-157, which are situated in the same troponin T peptide (residues 147–161) (Pearlstone *et al.*, 1976; Moir *et al.*, 1977). Fractions unadsorbed on phosphocellulose and possessing a high phosphorylase kinase activity phosphorylate only such troponin T sites, which are located in short tryptic peptides (Fig. 2b). The structure of one of these sites around serine-149–serine-150 is similar to that of the site phosphorylated in phosphorylase *b*. The fractions tightly adsorbed on phosphocellulose possessing troponin T kinase and phosvitin kinase activities phosphorylate only serine-1, located in the largest troponin T tryptic peptide (Fig. 2c). Summarizing the data obtained, it can be concluded that standard preparations of phosphorylase kinase are usually contaminated by troponin T kinase.

The possibility that the catalytic subunit of cyclic AMP-dependent protein kinase is responsible for the phosphorylation of serine-1 of troponin T is unlikely. Cyclic AMP-dependent protein kinase does not catalyse the phosphorylation of troponin T to any significant extent (Perry, 1979). Moreover purified troponin T kinase tightly adsorbed on phosphocellulose was not affected by the cyclic AMP-dependent protein kinase inhibitor described by Walsh *et al.* (1971).

Discussion

Phosphorylase kinase isolated from I-strain mice does not phosphorylate phosphorylase *b* at a significant rate, and at the same time phosphorylates a mixture of troponin I and troponin T at a rather high rate (Gross & Mayer, 1973). Moreover troponin T isolated from the I-strain mice is partially phosphorylated (Moir *et al.*, 1977). There is no difference in the pH profile for the phosphorylation of troponin I and troponin T mixture by phosphorylated and dephosphorylated preparations of phosphorylase kinase (Stull *et al.*, 1972). All these facts can be easily explained on the assumption that phosphorylase kinase preparations are contamin-

Fig. 2. The gel-filtration pattern of tryptic peptides of troponin T, phosphorylated by the original preparation of phosphorylase kinase (a), by fractions unadsorbed on phosphocellulose (fraction 5–15, Fig. 1) (b) and by fractions tightly bound to phosphocellulose (fractions 50–51, Fig. 1) (c).

Tryptic hydrolysate (0.2–0.5 ml) was put on a column (1.2 cm $\times$ 100 cm) of Bio-Gel P-4 and was eluted with 0.1 M-HCl. The A_{206} was monitored continuously and the radioactivity of fractions (4 ml) was measured by the Čerenkov method.

ated by troponin T kinase. The failure of Dickneite *et al.* (1978) to separate phosphorylase *b* and troponin T kinase activities is due to the fact that both enzymes are eluted from DEAE-cellulose under similar conditions (Dickneite *et al.*, 1978; Gusev *et al.*, 1978, 1980; Dobrovolskii, 1979; Kumon & Villar-Palasi, 1979).

Except for phosphorylase *b*, phosphorylase kinase catalyses autophosphorylation (Cohen, 1974) and phosphorylates troponin T, troponin I, glycogen synthetase and casein (DeLange *et al.*, 1968; Singh & Wang, 1979; De Paoli-Roach *et al.*, 1979). The sequence of the sites phosphorylated in phosphorylase *b*, the α -subunit of phosphorylase kinase, troponin I, glycogen synthetase and one of the troponin T sites (serine-149–serine-150) have some common features (Williams, 1976). At the same time the sequence of sites phosphorylated in casein and the sequence proximal to the phosphorylated serine-1 of troponin T differ from the sequence around the phosphorylated site of phosphorylase *b* (Dobrovolskii, 1979; Gusev *et al.*, 1980). It is therefore probable that the casein kinase activity present in phosphorylase kinase preparations is not due to phosphorylase kinase itself, but to the presence of the contaminating troponin T kinase.

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