Phosphorylation of the '37000 Component' of the Troponin Complex (Troponin-T)

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Most of the phosphorus present in the troponin complex, which on average contains 1 mol of P/80 000 g, is associated with the '37000 component' (0.6 mol of P/mol). The inhibitory protein and particularly the '37000 component', but not the calcium-binding protein, were phosphorylated when incubated with phosphorylase *b* kinase and [γ - 32 P]ATP.

Recent investigations in our laboratory (Wilkinson *et al.*, 1971, 1972) and by others (Ebashi *et al.*, 1971; Greaser & Gergely, 1971; Drabikowski *et al.*, 1971; Murray & Kay, 1971) have shown that the troponin complex consists of three distinct proteins. These are the calcium-binding protein of molecular weight 18000 with a high binding constant for Ca^{2+} , the inhibitory protein of molecular weight 23000 that inhibits the Mg^{2+} -stimulated adenosine triphosphatase activity of desensitized actomyosin and the '37000 component', the role of which is not yet well defined. Some investigators refer to these components as troponin-C, troponin-I and troponin-T respectively.

Bailey & Villar-Palasi (1971) first reported that the inhibitory protein was phosphorylated by ATP in the presence of a purified muscle protein kinase that required cyclic AMP. In extension of these findings Stull *et al.* (1972) also concluded that most of the phosphorylation occurred on the inhibitory protein when troponin B was incubated with phosphorylase *b* kinase.

It has been reported from this laboratory that the light-chain component of 18000 molecular weight present in skeletal myosin can be phosphorylated by phosphokinase preparations from rabbit skeletal muscle (Perrie *et al.*, 1972, 1973). As a development of this work we have investigated the ability of the enzyme systems that phosphorylate myosin to phosphorylate the troponin complex and its components.

Methods

Troponin. This was prepared by the methods of Ebashi *et al.* (1971), and it was fractionated into its components by the method of Wilkinson *et al.* (1972) or by chromatography on DEAE-cellulose (Greaser & Gergely, 1971) or on CM-cellulose equilibrated with 6M-urea–0.1M-sodium acetate buffer, pH4.7, containing 1mM-dithiothreitol (J. M. Wilkinson, personal communication). Calcium-binding protein was eluted with the starting buffer and the inhibitory protein and the '37000 component' were eluted

successively by application of a linear gradient to 0.2M-NaCl–6M-urea–0.1M-sodium acetate buffer, pH4.7. Components were rechromatographed and monitored by electrophoresis on a 10% polyacrylamide gel in 100mM-sodium phosphate buffer, pH7.0, containing 0.1% sodium dodecyl sulphate (Weber & Osborn, 1969). Tropomyosin was prepared as previously described (Wilkinson *et al.*, 1972).

Phosphokinase experiments. Phosphorylase *b* kinase was prepared as described by Krebs *et al.* (1964) and stored at -20°C in 50mM-sodium glycerophosphate buffer, pH7.0, containing 0.5mM- CaCl_2 and 1mM-dithiothreitol. The enzyme (0.1–0.5mg/ml) was incubated at 30°C with troponin or components (1–3mg/ml) in 10mM-Tris-HCl–10mM-sodium glycerophosphate buffer, pH8.6, containing 10mM-magnesium acetate, 0.05mM- CaCl_2 , 0.25mM-dithiothreitol, 1mM-theophylline, 10mM-NaF and 0.5mM-ATP containing [γ - 32 P]ATP of known specific radioactivity (The Radiochemical Centre, Amersham, Bucks., U.K.). The reactions were stopped with cold 5% (w/v) trichloroacetic acid. The precipitated protein was washed once with 2.5% (w/v) trichloroacetic acid containing 2.5mM-ATP followed by four washings with 2.5% trichloroacetic acid, and was finally dissolved in 0.5M-NaOH for protein determinations (Lowry *et al.*, 1951) with dry bovine serum albumin as standard. After incubation with [U - ^{14}C]ATP (The Radiochemical Centre) the precipitated protein was filtered off on to glass-paper discs and washed in the same way.

Counting of radioactivity. ^{32}P radioactivity was determined in 0.5M-NaOH by the Čerenkov method in a Philips model PW4510 liquid-scintillation analyser. ^{14}C radioactivity was counted on dried glass-paper discs in 10ml of scintillation fluid (Schaub & Perry, 1971). Polyacrylamide gels were dissolved in H_2O_2 (100vol.) at 80°C before measurements of radioactivity.

Total phosphate. This was determined on proteins precipitated with cold 5% trichloroacetic acid and washed several times with cold 2.5% trichloroacetic

acid by the method of Leloir & Cardini (1957). H_2O_2 (100 vol.) was used for decolorization, and protein was estimated by Nesslerization (Schaub & Perry, 1971).

Results and discussion

Troponin preparations, consisting of the calcium-binding protein, inhibitory protein and '37000 component' in approximately equimolar proportions, contained on average 0.98 ± 0.14 mol of P/80000 g of troponin. This phosphorus appeared to be covalently linked to the protein, for it was not removed by successive washings with 2% trichloroacetic acid or by prolonged dialysis against 1.25 mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate]. The purified components of the troponin complex contained the following amounts of total P/mol of protein: calcium-binding protein, 0.10 ± 0.03 mol; inhibitory protein, 0.05 ± 0.03 mol; '37000 component', 0.59 ± 0.24 mol.

On incubation of the whole troponin complex with phosphorylase *b* kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ there was significant incorporation of ^{32}P into the protein precipitated by 5% trichloroacetic acid and washed as described above under 'Methods'. The amount of ^{32}P incorporated into the phosphorylase *b* kinase was usually less than 5% of the total incorporation. The incorporation increased with time, approaching a maximum 0.2 mol of ^{32}P /80000 g of troponin after about 45 min. The troponin complex that had been phosphorylated in this manner was then separated into its components by sodium dodecyl sulphate gel electrophoresis. ^{32}P determinations on slices of the gel containing single bands indicated that the major radioactivity was associated only with the bands of mobility corresponding to the inhibitory protein and the '37000 component'. Minor amounts of radioactivity were associated with a band of mobility corresponding to a molecular weight of about 50000, at which position the phosphorylase *b* kinase migrated.

In all experiments involving phosphorylation of the troponin complex the amount of radioactivity in the 23000 component band was consistently lower than that in the band corresponding to the '37000 component'. As it was not possible to determine accurate specific radioactivities of the components separated by this method, 150 mg of troponin was phosphorylated in the same way and then fractionated into its three components by chromatography on CM-cellulose. No significant radioactivity could be detected in the calcium-binding protein, whereas the inhibitory protein contained 0.06 mol of ^{32}P /mol and the '37000 component' 0.2 mol of ^{32}P /mol. The latter fraction was a single band on sodium dodecyl sulphate gel electrophoresis. The inhibitory protein isolated in this experiment was about 90% pure and the minor bands present were of lower molecular weight than 23000, probably owing to endogenous

catheptic activity, to which the protein is particularly susceptible (Wilkinson *et al.*, 1972). It was concluded that the radioactivity ascribed to the inhibitory protein was not due to contamination with the '37000 component'.

Pure samples of the '37000 component' (Plate 1c) and preparations of the inhibitory protein (Plate 1b) possessing less than 10–15% of lower-molecular-weight fragments (see above) became radioactive when incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and phosphorylase *b* kinase (Fig. 1). Neither the calcium-binding protein (Fig. 1) nor a purified sample of tropomyosin showed any significant incorporation of radioactivity when incubated under the same conditions. Preparations of the '37000 component', which on average contained 0.6 mol of P/mol, incorporated an additional 0.1–0.5 mol of ^{32}P /mol. This incorporation, which was somewhat variable between preparations, possibly because of the partial solubility of the '37000 component' under the incubation conditions, suggested that phosphorylation proceeded until 1 mol of P was present/mol of '37000 component'. We conclude that most of the covalently bound phosphorus in troponin is probably due to the '37000 component'. The inhibitory protein did not incorporate more than 0.25 mol of ^{32}P /mol in this type of experiment, which is surprising in view of its low initial phosphate content.

When $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was replaced by $[\text{U}\text{-}^{14}\text{C}]\text{ATP}$ in the phosphorylating system no significant radioactivity could be detected in the troponin complex or the separately incubated '37000 component'. This confirmed what would be expected from the known specificity of the phosphokinase, namely that neither adenine nor ribose was transferred to the acceptor proteins.

This evidence strongly suggests that the terminal phosphoryl group of ATP was transferred to the '37000 component' and the inhibitory protein to give a phosphate ester, presumably on a serine or a threonine residue. The presence of the phosphate ester link in both of these components isolated from the troponin complex previously phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was confirmed by the progressive liberation of ^{32}P with time (Fig. 1b) when each was incubated with a purified sample of the *Escherichia coli* alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

Incubation of the troponin complex in the presence of 83 mM-sodium glycerophosphate buffer, pH 6.0, containing 0.33 mM-EGTA and 0.033 mM-cyclic AMP with a crude protein kinase preparation obtained by the method of Walsh *et al.* (1968) gave less than 10% of the incorporation of radioactivity obtained with phosphorylase *b* kinase. Under these conditions this preparation produced some incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into the 18000-molecular-weight light chain of myosin (Perrie *et al.*, 1973).

EXPLANATION OF PLATE I

Sodium dodecyl sulphate gel electrophoresis of separated troponin components used for phosphorylation and dephosphorylation (Fig. 1)

Protein was applied to 10% polyacrylamide gels as described in the text under 'Methods'. (a) 20 μ g of calcium-binding protein; (b) 60 μ g of inhibitory protein; (c) 40 μ g of '37000 component'. The electrophoresis runs were carried out on different occasions.

Fig. 1. Phosphorylation and dephosphorylation of the components of the troponin complex

(a) Incorporation of ^{32}P into the separated components of the troponin complex. The proteins illustrated in Plate 1(a), 1(b) and 1(c) were incubated with phosphorylase *b* kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (see the text under 'Methods'). $\circ$, '37000 component'; Δ , inhibitory protein; $\bullet$, calcium-binding protein. (b) Dephosphorylation of components isolated from the troponin complex. Troponin was phosphorylated as the complex and fractionated into components (see the text under 'Methods'). Proteins (0.2 mg/ml) were incubated with *Escherichia coli* alkaline phosphatase (0.7 $\mu\text{g}/\text{ml}$) at 30°C in 0.3 M-NaCl-30 mM-Tris-HCl buffer, pH 8.2. $\circ$, '37000 component'; Δ , inhibitory protein.

In the apparent preferential phosphorylation of the '37000 component' our findings differ from those of Stull *et al.* (1972), most of whose studies were on troponin B preparations, which normally contain considerable amounts of the '37000 component' (Wilkinson *et al.*, 1972). Possibly the enzyme preparations used in the two laboratories contained different proportions of phosphokinases with different specificities for the inhibitory protein and the '37000 component'. Since our work was completed Pratje & Heilmeyer (1972) have reported that, when the troponin complex was phosphorylated with a protein kinase preparation, a protein of 39000 molecular weight, which probably corresponds to our '37000 component', incorporated considerably more phosphate than did the inhibitory protein.

The results reported here, together with previous reports of the phosphorylation of the light-chain component of myosin (Perrie *et al.*, 1972, 1973), indicate that at least three protein components of the myofibril can be phosphorylated by phosphorylase *b* kinase. By analogy with other systems in which enzymes are phosphorylated it might be assumed that the process is concerned with the regulation of the

enzymic activity of the myofibril. As yet, however, there are no clear indications of the mechanisms involved or indeed whether they have any regulatory role at all.

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