PHOSPHORYLATION OF A MYOFIBRILLAR PROTEIN OF M_r 150 000 IN PERFUSED RAT HEART, AND THE TENTATIVE IDENTIFICATION OF THIS AS C-PROTEIN

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1. Introduction

The increase in contractility which occurs on exposure of cardiac muscle to catecholamines is thought to be mediated by an increase in the intracellular concentration of cyclic 3′,5-AMP [1,2]. It is proposed that this increase activates cyclic 3′,5′-AMP-dependent protein kinase, resulting in the phosphorylation of membrane and myofibrillar proteins (reviewed in [3,4]). The phosphorylation of a number of cardiac myofibrillar proteins has been studied in detail both in vitro and in vivo. The inhibitory subunit of troponin (troponin-I) can be phosphorylated in vitro by cyclic AMP-dependent protein kinase [5,6], and in perfused heart in response to agents which elevate cyclic 3′,5′-AMP [7,8]. This results in a decrease in calcium sensitivity of both the adenosine triphosphatase of isolated myofibrils [9,10] and tension development in skinned cardiac fibres [11,12]. It is possible that this is related to the decrease in relaxation time observed on treatment of cardiac muscle with catecholamines [3,4]. The P-light chain of myosin is also phosphorylated in cardiac muscle, although the light chain kinase is a Ca^{2+}-dependent enzyme and is not activated by cyclic AMP [13,14]. The level of phosphorylation of the P-light chain in perfused hearts does not appear to be affected by catecholamines or increased Ca^{2+} however [15,16], and is probably not involved with short-term regulation of contractility.

C-protein is a protein of M_r 140 000–150 000 which is associated with myosin in the thick filaments of striated muscle [17,18]. Use of antibodies to C-protein on intact myofibrils showed that it was located at a spacing of 43 nm along the thick filament [19]. C-protein will bind to various fragments from the tail region of myosin [20], and may possibly be concerned with either the structural integrity or the assembly of the thick filament [18,21]. Recently it has been shown that at physiological ionic strength C-protein from rabbit skeletal muscle caused a mild activation of actin-activated myosin ATPase [22]. However, the precise function of C-protein is at present still unknown.

When cardiac myofibrils were incubated with cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP, significant incorporation of ^{32}P occurred in only 2 proteins [9]. One of these was troponin-I, the other had M_r ~150 000. Here we show that in rat hearts perfused with $^{32}P_i$, a protein of M_r 150 000 was phosphorylated, and that this phosphorylation was increased 5–6-fold by exposure of the hearts to adrenaline for 20 s. Evidence is presented suggesting that this phosphorylated protein is C-protein.

2. Methods

2.1. *Phosphorylation of proteins in perfused heart*

Hearts from female Wistar rats (200–220 g) were perfused by the Langendorff technique with $^{32}P_i$ for 15 min (spec. radioact. 2 Bq/pmol) as in [7,16]. Hearts were then perfused with 5 μM adrenaline for up to 60 s before being freeze-clamped and powdered at −196°C.

Samples for polyacrylamide gel electrophoresis were prepared essentially as in [23]. Frozen tissue (200 mg) was homogenised in 2 ml 8 M urea, 100 mM glycine, 5 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) (pH 1.5). Insoluble protein was removed; the solubilised protein precipitated with trichloracetic acid and dissolved in buffer for electro-

phoresis on polyacrylamide by the Laemmli method [24]. After electrophoresis, protein was detected with Coomassie brilliant blue and protein-bound ^{32}P measured by densitometric scanning after autoradiography of the gel. The autoradiographs were within the range of proportionality between absorbance and radioactivity.

The specific radioactivity of [γ-^{32}P]ATP in frozen hearts was measured as in [25].

2.2. *Preparation of 150 000* M_r *protein*

This was prepared by a modification of the method in [18]. Following perfusion with $^{32}P_i$ as above, two rat hearts (unfrozen) were finely chopped and homogenised by hand in 12 ml 50 mM KP_i, 70 mM NaF, 5 mM EDTA, 0.3 M sucrose, 0.1 mM PMSF (pH 7.0) and a crude myofibrillar preparation made by washing in 1% Triton X-100 [15]. All extraction procedures were carried out at 0–4°C. The myofibrils were extracted in 7.5 ml 0.15 M KP_i, 10 mM EDTA (pH 7.0) for 15 min, and the solubilised protein dialysed overnight against 0.15 M KP_i, 1 mM EDTA, 2 M urea (pH 7.5). The protein was chromatographed on a column (5 cm × 1 cm) of DEAE–Sepharose CL-6B (Pharmacia). Protein fractions were analysed by polyacrylamide gel electrophoresis and autoradiography.

3. Results and discussion

3.1. *Phosphorylation of the 150 000* M_r *protein in perfused heart*

Fig.1 shows the distribution of ^{32}P in proteins after perfusion of hearts with $^{32}P_i$. Three major phosphorylated bands of M_r 150 000, 27 000 and 19 000 can be identified. The 27 000 M_r protein co-migrated with purified troponin-I, and the 19 000 M_r protein with the P-light chain of myosin. In control perfusions only the P-light chain contained an appreciable amount of ^{32}P which was unchanged after exposure of the hearts to adrenaline [16]. Both troponin-I and the 150 000 M_r protein contained small amounts of ^{32}P in control perfusions, which increased considerably on exposure to adrenaline.

Fig.2 show the time courses of increases in aortic pressure and phosphorylation of the 150 000 M_r protein and troponin-I following perfusion with 5 μM adrenaline. There was an ~5–6-fold increase in ^{32}P content of the 150 000 M_r protein during 22 s perfusion with adrenaline, which was in parallel with the increase in contraction. The ^{32}P content of troponin-I also increased 5–6-fold over the same time-course, a result in agreement with earlier studies [26]. After 22 sec of exposure to adrenaline approximately equal amounts of ^{32}P had been incorporated into troponin-I and the 150 000 M_r protein.

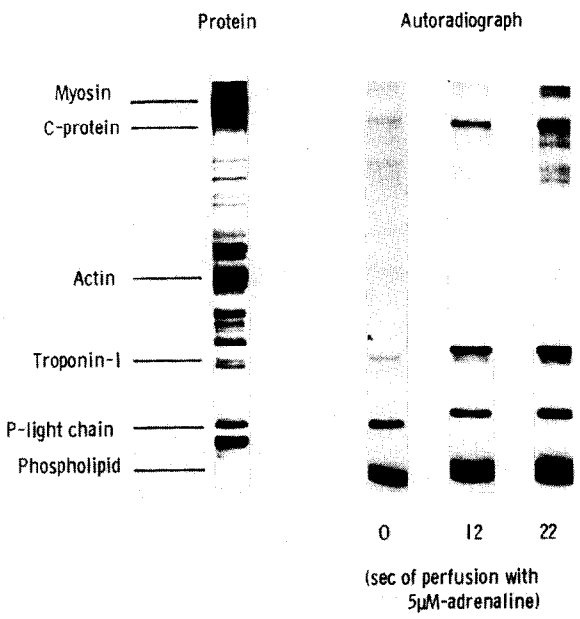

Fig.1. Distribution of protein and ^{32}P in rat heart after perfusion with $^{32}P_i$. Whole heart extracts were prepared as in section 2 and subjected to electrophoresis [24] on a 6–12.5% gradient of polyacrylamide.

3.2. *Identity of the 150 000* M_r *protein*

The 150 000 M_r protein remained attached to rat heart myofibrils during extensive washing with Triton X-100 and low ionic strength buffers. A preparation of crude myosin made by extracting a rat heart homogenate with Guba-Straub solution [27], also contained the 150 000 M_r protein, indicating a close association between these proteins. C-protein of skeletal muscle is myofibrillar, and is also extracted along with myosin under the above conditions [18].

In order to show that the 150 000 M_r protein could be C-protein, further purification was carried out using a method for the preparation of C-protein from skeletal muscle [18]. An extract of myofibrils was prepared and chromatographed on DEAE–Sepharose as in section 2. Fig.3 shows the protein elution patterns for extracts from both rat cardiac

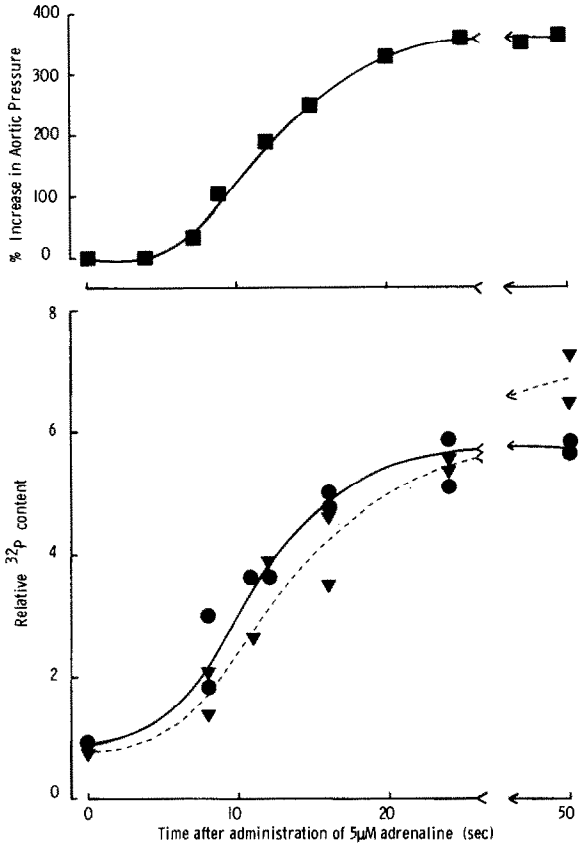

Fig.2. Time courses of changes in aortic pressure (■) and phosphorylation of the 150 000 M_r protein (●) and troponin-I (▼) following perfusion with 5 μM adrenaline. The ^{32}P incorporation into the proteins was corrected by the measured [γ-^{32}P]ATP specific radioactivity of each heart, and is expressed relative to the ^{32}P incorporation in the 150 000 M_r protein in perfusions without adrenaline.

and skeletal muscle. There was a small peak of unbound protein, and a large peak which eluted with 0.5 M KCl. When the two protein peaks from cardiac muscle were analysed by polyacrylamide gel electrophoresis, peak I was enriched in the 150 000 M_r protein, but also contained some myosin and thin filament proteins (fig.4). Peak II contained very little of the 150 000 M_r protein, and was mainly undissociated actomyosin. Peak I from rat skeletal muscle contained predominantly a protein of M_r 140 000 (not shown), which is identical to the elution pattern observed with C-protein from rabbit skeletal muscle [18]. The majority of ^{32}P in peak I prepared from hearts perfused with $^{32}P_i$ and adrenaline migrated in the same place as the protein of M_r 150 000 (fig.4).

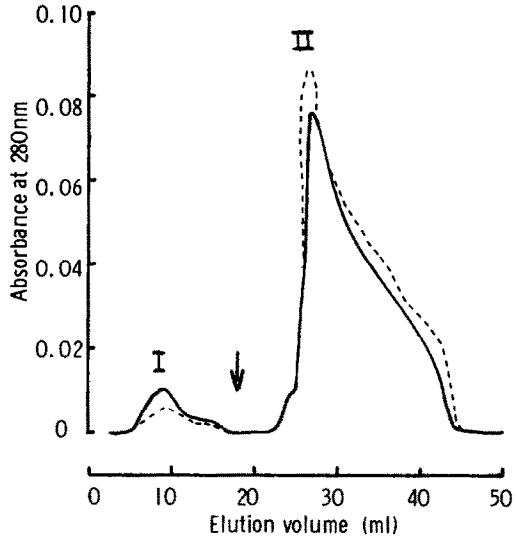

Fig.3. Separation of 150 000 M_r protein by chromatography on DEAE–Sepharose. Cardiac (——) or skeletal (– – –) myofibrils were extracted and chromatographed as in section 2. The arrow indicates the addition of 0.5 M KCl to the elution buffer.

We therefore tentatively identify the protein of M_r 150 000 as C-protein. Peak I also contained other phosphorylated proteins, the major one being troponin-I. Several of the others appear to be proteolytic fragments of C-protein, as aged preparations show a decrease of ^{32}P-labelled protein of M_r 150 000, and an increase of ^{32}P in the lower M_r proteins.

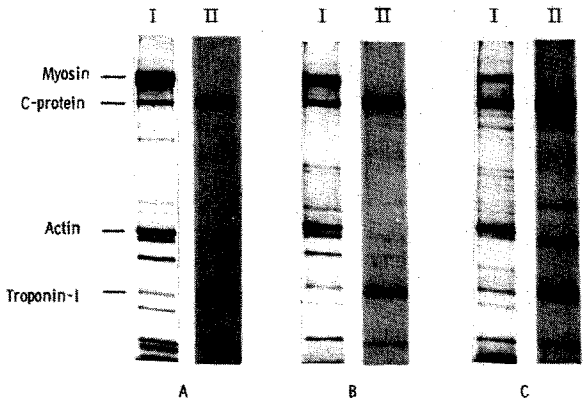

Fig.4. Distribution of protein (I) and ^{32}P(II) in fractions from stages during the preparation of the 150 000 M_r protein: (A) crude myofibrillar fraction; (B) extract of myofibrils in 0.15 M KP_i, 10 mM EDTA (pH 7.0); (C) peak I from the DEAE–Sepharose chromatography.

It appears from these results that C-protein in heart has a higher M_r than in skeletal muscle. In addition, cardiac C-protein dissociates less easily from other myofibrillar proteins than C-protein from skeletal muscle, and is thus more difficult to purify.

3.3. *General discussion*

Previous results [9] showed that a protein of M_r 150 000 could be phosphorylated in myofibrils from beef and rat heart by cyclic AMP-dependent protein kinase. Here we show that this protein is probably C-protein and is phosphorylated in intact heart in response to adrenaline over the same time course as troponin-I. Troponin-I is known to be phosphorylated in vivo by cyclic AMP-dependent protein kinase [28]. These results suggest that C-protein is also phosphorylated in vivo by the same kinase.

After stimulation of hearts with adrenaline the amount of ^{32}P in C-protein was very similar to that in troponin-I (fig.2). It is possible to calculate the molar ratio of ^{32}P to C-protein in these hearts if it is assumed that the ratio of troponin to C-protein is the same in heart as in skeletal muscle. In skeletal muscle the ratio of troponin: C-protein is ~5:1 (G. Offer, personal communication). After 20 s perfusion of rat heart with adrenaline, troponin-I contains 1 mol ^{32}P/mol [26]. From these assumptions and the results of fig.2 it appears that in control conditions C-protein contains ~1 mol ^{32}P/mol, and that this increases to 5 mol/mol with adrenaline. This is obviously a preliminary calculation, and requires verification by direct measurement of covalentlybound phosphate in purified C-protein. Work is also in progress to confirm that this 150 000 M_r protein is cardiac C-protein.

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