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THE HEAT LABILE PHOSPHATASE MODULATOR (INHIBITOR-2) COMPLEX FROM RABBIT SKELETAL MUSCLE

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The heat stable phosphatase modulator protein (inhibitor-2) has been shown to play a crucial role in the reversible ATP, Mg-dependent activation of a multisubstrate protein phosphatase. The modulator activity is acid and heat stable and resides in a small asymmetrical protein which, after boiling migrates in sucrose density gradient centrifugation with a molecular weight of 17K. The present report shows that in unboiled rabbit skeletal muscle preparations all the modulator activity is found associated with a heat labile protein component, which imposes an important regulatory feature on the heat stable activity. The heat labile complex migrates in sucrose density gradient centrifugation as a M_r=70K protein.

Two heat stable proteins have been implicated in the regulation of the cellular phosphorylase phosphatase activity (1-3); they have been termed inhibitor-1 and -2. Inhibitor-1 is phosphorylated by the cyclic AMP-dependent protein kinase and is responsible for the decrease in the cellular phosphorylase phosphatase activity expected and observed after adrenalin release (3). The heat stable inhibitor-2 has recently been identified as a modulator protein, which can regulate a multisubstrate protein phosphatase activity in two ways: it converts the phosphatase to an inactive form and is also absolutely required for the F_A-ATP,Mg-dependent reactivation of this inactivated enzyme (4). The physiological importance of these heat stable proteins has been questioned (5-8), although their activities have been measured in unboiled preparations (9-10) and inhibitor-1 has been partially purified without including a boiling step in the isolation procedure (9). The present report

Abbreviations: F_A, activating protein of the ATP,Mg-dependent phosphatase; SDS, sodium dodecyl sulfate.

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indicates that although the modulator (inhibitor-2) activity is associated with an acid and heat stable protein (M_r=17K) it is present in unboiled tissue preparations as a heat labile, higher molecular weight protein complex. The heat stable component by itself lacks some regulatory features.

MATERIALS AND METHODS

Materials and methods are essentially as described in previous reports (11-13). The activity of the ATP,Mg-dependent phosphatase was measured after a 10 min preincubation at 30°C with 0.1 mM ATP, 0.5 mM Mg^{2+} and the minimal amount of F_A, required for full activation of the ATP,Mg-dependent phosphatase under these conditions. The phosphatase activity unit is defined as the amount of enzyme which releases 1 nM of (^{32}P)phosphate/min at 30°C from ^{32}P-labeled phosphorylase a (2 mg/ml). The assay time is 5 min.

Heat stable modulator (inhibitor-2) was purified to homogeneity from rabbit skeletal muscle (13). One unit of modulator, measured as phosphatase inhibitor, decreased the phosphatase activity by 1 unit when included in the assay of 30 milliunits of phosphatase enzyme. The phosphatase enzyme used was the M_r=35K enzyme isolated essentially according to (14) from rabbit skeletal muscle: its specific activity was about 50,000 units per mg protein. The modulator was commonly preincubated with the phosphatase for 5 min at 30°C before determination of the phosphatase activity: When unboiled preparations were used for determination of the modulator (inhibitor) activity, care was taken to correct for the endogeneous phosphatase activity of the preparation.

Sucrose density gradient centrifugations were performed in a Beckman SW50-1 rotor at 4°C, 49,000 rpm for 17 hrs, using 5 to 20% sucrose made in 20 mM Tris-HCl, 1 mM dithiothreitol pH 7.0 (buffer A). Tentative molecular weights were calculated (11) using ^{14}C-ovalbumin as internal marker protein (M_r=45K).

RESULTS AND DISCUSSION

An extract was prepared from 1.5 kg of rabbit skeletal muscle (12). The cytosolic proteins obtained after centrifugation in a Beckman Type 19 rotor, at 19,000 rpm and 4°C, for 3 hours were absorbed batchwise onto one liter of DEAE-Sephadex A-50 equilibrated in buffer A. The resin was washed with 0.2 M NaCl in buffer A on a funnel, subsequently packed into a (5x40 cm) column, and further washed with this buffer until the absorbance at 280 nm was below 0.05. The adsorbed proteins were then eluted with a salt gradient (2x500 ml) from 0.2 M to 0.4 M NaCl in buffer A, and assayed for total phosphatase

activity (after a preincubation with F_A-ATP,Mg) and for modulator activity (measured after boiling as inhibition on the M_r=35K phosphatase catalytic unit). This ion exchange chromatography is the usual first step in the purification of both the ATP,Mg-dependent protein phosphatase (12) and the modulator protein (13), and the activity obtained in these pooled fractions can rightly be taken as 100%. It was noted that both activities coincided perfectly in the gradient elution (not shown), and the ratio of total phosphatase (%) to modulator activity (%) was arbitrarily taken as 1.

The pooled fractions were concentrated by precipitation with solid ammonium sulfate (30-50% saturation) and extensively dialyzed against buffer A; about 90% of both activities were recovered after this concentration step (Table 1). It was observed that by this time most of the phosphorylase phosphatase activity was already present in its inactive ATP,Mg-dependent form (not shown). The observation that 92% of the

TABLE 1. Copurification of modulator and phosphatase activity

preparations	Total modulator activity (A) %	Total phosphatase activity (B) %	Ratio $\frac{B}{A}$
DEAE-Sephadex A-50 pooled fractions (step 1)	100	100	1.0
30-50% Ammonium sulfate fraction (step 2)	92	94	1.0
Blue Sepharose CL-6B breakthrough fraction (step 3)	80	75	0.9
Ultrogel ACA 34 pooled fractions (M_r=120K)	62	65	1.0
Sucrose density gradient pooled fractions (M_r=70K)	65	72	1.1

The total phosphatase activity was measured after an incubation with F_A-ATP,Mg, necessary to activate the inactive phosphatase present in the different preparations used. The total modulator activity was measured after boiling.

modulator activity was precipitated by ammonium sulfate at 50% saturation, distinguishes it from boiled modulator preparations which require more than 80% ammonium sulfate saturation for quantitative recovery in the precipitate (not shown). The next step in the purification of modulator is usually a heat treatment after which the heat stable protein is quantitatively adsorbed onto a Blue Sepharose CL-6B resin (13). When the boiling step is omitted, the modulator activity does not stick to the Blue Sepharose CL-6B resin, and is recovered, together with the inactive phosphatase enzyme in the breakthrough of the column. The activity ratio between these two proteins is again not changed (Table 1). Copurification of some heat stable inhibitory activity with the inactive ATP,Mg-dependent phosphatase activity has been reported in some of our previous publications (4,11).

The breakthrough proteins from the Blue Sepharose CL-6B column were concentrated by dialysis against 10% polyethyleneglycol 6000 (13) and the molecular weight of the proteins which exhibit modulator and/or phosphatase activity was determined. This was done either by gel filtration on Ultrogel ACA-34 columns, or by sucrose density gradient centrifugation. Gel filtration gave a M_r=120K for a protein peak which had both activities, and the ultracentrifugation experiment suggested a M_r=70K protein which again harboured modulator and phosphatase activity (Fig. 1A and 1B respectively). The phosphatase-modulator activity ratio remained the same (Table 1). When an identical sample was first boiled for 5 min and subsequently subjected to the same ultracentrifugation the modulator activity was found to migrate exclusively as a M_r=17K protein(Fig. 1C). This M_r=17K modulator was identical to the purified modulator protein characterized in (13) in all criteria tested: acid and SDS stability, M_r=35K in SDS-polyacrylamide gel electrophoresis and M_r=65K in gel filtration (not shown).

These results clearly indicate that the modulator activity associated with a M_r=17K heat stable, asymmetrical protein is present in a higher molecular weight complex, which is disrupted by a boiling step and therefore is likely to contain heat labile components. These heat labile components may

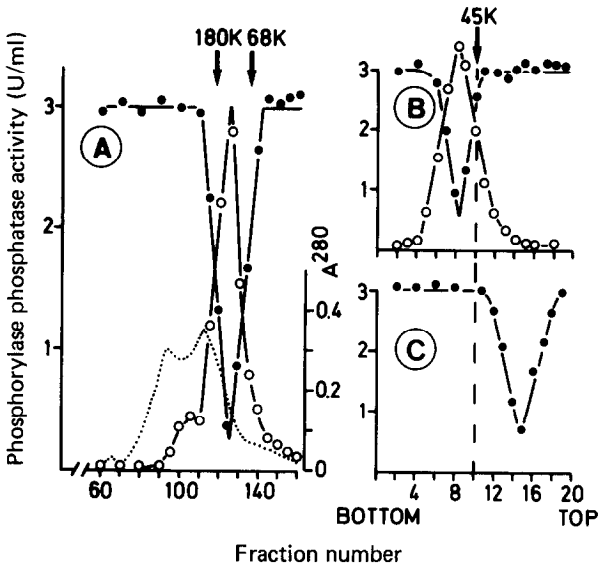

Fig. 1. *Migration of ATP,Mg-dependent phosphatase and modulator activity in gel filtration and sucrose density gradient centrifugation.*
Panel A shows the elution profile of total phosphorylase phosphatase- (o) and modulator (inhibitor-2)(•) activity on a Ultrogel ACA 34 column (2.5 x 90 cm). The dotted line represents absorbance at 280 nm. The elution of the marker proteins: phosphorylase b (180 K) and bovine serum albumin (68K) are indicated by the arrows. Panel B and C represent 5 to 20% sucrose density gradient centrifugation experiments with ^{14}C-ovalbumin (M_r=45K) as internal marker protein. Panel B shows the migration of total phosphatase activity (o) (2 units of step 3 enzyme); the modulator activity (•) is assayed after boiling the individual fractions. Panel C shows the migration of modulator (•) when an identical sample is first boiled and subsequently applied to the sucrose gradient. The fractions are assayed as such for modulator activity.

have ATP,Mg-dependent phosphatase activity since the unboiled modulator and the ATP,Mg-dependent (or total-) phosphatase activity copurify in a constant activity ratio in several separation steps (Table 1). This heat labile complex has a M_r= 120K on gel filtration and a M_r=70K in ultracentrifugation, which may either reflect an asymmetrical character of the complex or a dissociation by the centrifugal force into monomeric units.

The phosphatase activity of the M_r=35K catalytic subunit is decreased in a time and dose dependent way by the unboiled (step 3)-preparation. This effect can be completely abolished by a subsequent F_A-ATP,Mg-dependent activation step, indicating that an inactive ATP,Mg-dependent enzyme form was produced (Fig. 2). After boiling, the same amount of sample decreased this phosphatase activity in a more rapid, but partially ir-

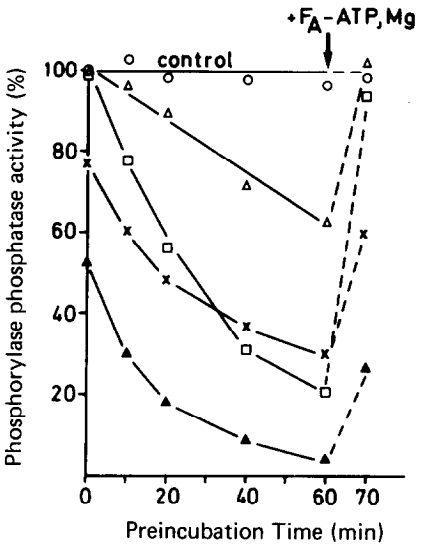

Fig. 2. *Modulation of phosphatase activity by boiled and unboiled modulator preparations.*
Inhibition of 30 milliunits of M_r=35K phosphatase catalytic subunit by 1.5 µg (□), 0.5 µg (Δ,▲) or 0.25 µg (x), of the (step 3)-preparation without (Δ,□) or after (x,▲) boiling; in the control samples (o) no additions were made. At the 60 min time point, F_A-ATP,Mg was added to reactivate the enzymes in a further 10 min preincubation. A 2 min phosphatase assay was done after appropriate dilution of each sample.

reversible way, suggesting that a non-specific inhibition of the phosphatase was taking place instead of a reversible inactivation to the ATP,Mg-dependent enzyme form. This irreversible inhibition observed by boiled modulator preparations (4,15) may be of no physiological importance, and recombination of active phosphatase catalytic unit with boiled modulator (inhibitor) preparations (16) may not result in the formation of phosphatase enzymes which represent or resemble the *in vivo* occurring enzyme species.

The purified ATP,Mg-dependent protein phosphatase contains a M_r=70K protein staining band on SDS-gels. Its function has not been clearly established: we have suggested before (4) that this protein could represent the inactive catalytic subunit, but it is also possible, and may be more likely that it constitutes a heat labile binding protein for the phosphatase moculator.

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