CONVERSION OF ACTIVE PROTEIN PHOSPHATASE TO THE ATP-Mg-DEPENDENT ENZYME FORM BY INHIBITOR-2

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1. Introduction

Brandt et al. [1,2] made the original observation that liver and muscle extracts contain heat-stable, trypsin-labile proteins which inhibit phosphorylase phosphatase. The regulatory role of these heat-stable proteins in the control of phosphatase activity was accentuated when two heat-stable inhibitors, termed inhibitor-1 and -2 were discovered and the inhibitory capacity of inhibitor-1 demonstrated to depend upon its phosphorylation by the cyclic AMP-dependent protein kinase [3,4]. Heat-stable phosphatase inhibitors have since been identified in a variety of animals and tissues [5–10] and purified to homogeneity from rabbit muscle (inhibitor-1, -2) [8,10] and rabbit liver [7]. The implication of inhibitor-1 in the hormonal regulation of glycogen metabolism has been clearly demonstrated [11–13].

We have reported the purification and characterisation of an ATP-Mg-dependent protein phosphatase system from rabbit muscle [14–19]: an inactive multifunctional protein phosphatase (F_C) was shown to be activated by another protein factor (F_A) in the presence of ATP-Mg ions (without phosphorylation). This report provides evidence that purified inhibitor-2 preparations [10] have the capacity to reverse the F_A and ATP-Mg-mediated activation.

2. Materials and methods

Most materials and methods have been described in [14,17]. The ATP-Mg-dependent phosphatase activity was commonly measured after a 10 min preincubation at 30°C with the activating protein F_A, 0.1 mM ATP and 0.5 mM Mg-acetate. One unit of phosphatase activity releases 1 nM [^{32}P]phosphate/min at 30°C from ^{32}P-labelled phosphorylase a (2 mg/ml).

Inhibitor-2 was purified to homogeneity from rabbit muscle according to [10]: specific activity was 80 000 U/mg. One unit of inhibitor-2 measured according to [10], decreased the activity of the ATP-Mg-dependent phosphatase [14] by 50%. The ATP-Mg-dependent phosphatase and protein phosphatase-1 have the same sensitivity towards the heat-stable protein inhibitors [19].

Purified rabbit muscle inhibitor-1 (phosphorylated) was a generous gift of Dr P. Cohen, University of Dundee.

Rabbit muscle protein phosphatase, F_C (10 000 U/mg) and the activating protein F_A (5000 U/mg) were purified according to [1] and [2], respectively.

TPCK-treated trypsin was purchased from Worthington Biochemical Co. (England) and soybean trypsin inhibitor from Sigma Chemical Co. (USA).

3. Results and discussion

3.1. *Preparation of trypsin-treated activated F_C-enzyme*

About 20 000 U F_C (2 mg protein) were fully activated by F_A and ATP-Mg and subsequently incubated at 30°C for 5 min with 0.2 mg TPCK-treated trypsin/ml. The proteolysis was stopped by the addition of 1.0 mg soybean trypsin inhibitor/ml and the 'irreversibly activated F_C-enzyme' applied to a (1 × 1 cm) polylysine–Sepharose 4B column equilibrated in a 20 mM Tris, 0.5 mM dithiothreitol pH 7

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buffer. F_A, ATP-Mg, the [trypsin–trypsin inhibitor] complex and residual free trypsin inhibitor could be completely washed out from the resin with a 0.2 M NaCl rinse and the phosphatase activity was subsequently eluted with 0.5 M NaCl in the same buffer, as in [14,17,18]. The pooled fractions were concentrated by dialysis against 20 mM Tris, 0.5 mM dithiothreitol, 50% glycerol (pH 7) for 1 h. The phosphatase activity measured with or without an F_A and ATP-Mg preincubation was the same (>9000 U/mg protein), indicating that the F_C-enzyme was still fully activated.

3.2. *Inhibitor-2 mediated conversion to the inactive ATP-Mg-dependent enzyme form (F_C)*

When an appropriately diluted sample of the trypsin-treated, activated F_C (~3 nM or 2 U/ml) was incubated at 30°C for up to 40 min, no reversal to the inactive F_C-form was noticed, as reported [17,18] when activated F_C was used without trypsin treatment. The phosphatase activity at $t = 0$ was taken as 100% (fig.1). When 0.1–0.4 μg inhibitor-2/ml (5–20 nM or 8–32 U/ml) was included in the preincubation, a time-dependent conversion to the inactive F_C-enzyme was observed (fig.1). It should be mentioned however that when an excess of inhibitor-2 was added to the preincubation mixture, we were not able to fully reactivate the phosphatase to the original level by an F_A and ATP-Mg preincubation. The time dependency of the inhibitor-2-mediated inhibition has also been observed using partially purified preparations [20] of the glycogen-bound phosphatase (500 U/mg) isolated from dog liver (not shown).

When phosphorylated inhibitor-1 was used instead, we observed a concentration-dependent decrease of the phosphatase activity, which was instantaneous (not time-dependent): no reactivation of the enzymic activity by F_A and ATP-Mg was possible (not shown). These results suggest that whereas phosphorylated inhibitor-1 shows all the characteristics of a real inhibitory protein, whose effect can be abolished by dilution [9], the inhibitor-2 protein can decrease the enzymic activity by converting the active phosphatase to the inactive ATP-Mg-dependent F_C-form. The effect of inhibitor-2 could not be reversed by dilution. A detailed kinetic analysis of the mode of action of inhibitor-1 has been made using a highly purified preparation of a multifunctional protein phosphatase [9]: the inhibition was of a simple non-competitive nature. The inhibition by inhibitor-2 has been described as mixed non-competitive [10].

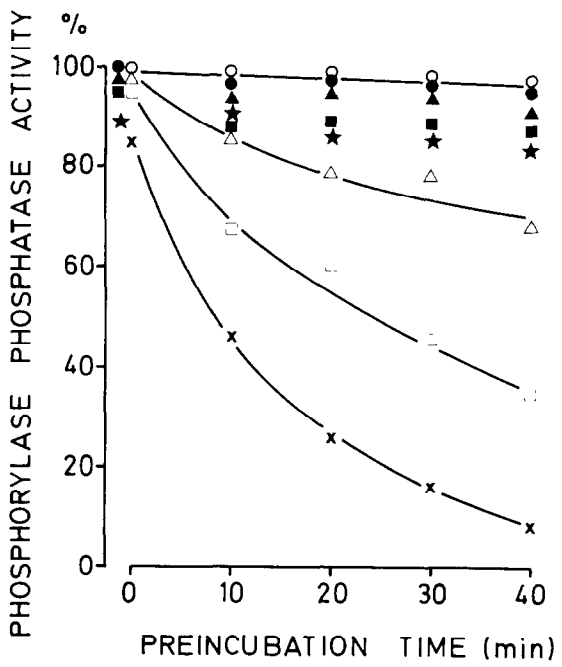

Fig.1. Time-dependent reversal of the F_C-activation after trypsin-treatment and polylysine–Sepharose 4B chromatography by preincubation at 30°C in the absence (○,●) or presence of 0.1 (△,▲), 0.2 (□,■) or 0.4 (x,★) μg inhibitor-2/ml, respectively. At the various time points, the preparations were assayed for total phosphatase activity (after a preincubation with F_A and ATP-Mg) and for activated F_C (without (●,▲,■,★) an additional preincubation with F_A and ATP-Mg) (○,△,□,x).

Whereas inhibitor-1 has been clearly implicated in the hormonal control of glycogen metabolism, by virtue of the regulation of its activity by the cyclic AMP-dependent protein kinase [11–13] no specific role has been attributed to inhibitor-2 in the regulation of the phosphatase activity. The concentration of a heat-stable protein inhibitor of phosphatase activity has been shown to vary with the diabetic state of rat livers, and to be under insulin control [21]. However the exact nature of the inhibitor involved in these experiments was not specified.

4. Conclusions

The mechanism of interconversion between the inactive ATP-Mg-dependent phosphatase (F_C) and the spontaneously active enzyme has been explained as being the result of a metal activation [17,18]. A phosphate incorporation into F_C was excluded although

the activating protein (F_A) has been shown to exhibit synthase kinase activity. This report shows that the reversal of this activation can be promoted by the heat-stable phosphatase inhibitor-2. This confirms the non-covalent nature of the phosphatase interconversion and provides evidence for a specific role of inhibitor-2 in the inactivation of the phosphatase. The irreversibility of the F_C activation after trypsin treatment may have been the result of the proteolytic destruction of the inactivating protein. F_C has been reported to contain heat-stable phosphatase inhibitory activity [17].

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