ON THE MECHANISM OF ACTIVATION OF RABBIT SKELETAL MUSCLE ATP-Mg-DEPENDENT PROTEIN PHOSPHATASE

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1. Introduction

An inactive form of protein phosphatase (F_C) has been purified to near homogeneity from rabbit skeletal muscle [1]. The enzyme can be activated by another protein factor (F_A) in the presence of ATP and Mg^{2+} without demonstrable incorporation of P_i into the protein [1,2]. The activated phosphatase has a broad substrate specificity [3,4]. The two protein factors (F_A,F_C) which constitute the ATP-Mg-dependent phosphatase system have been demonstrated in a variety of mammalian tissues [5,6] and can be used interchangeably from the different sources. It has been suggested that F_C may represent the inactive form of the physiologically active protein phosphatase, responsible for the major dephosphorylation reactions of the enzymes involved in the hormonal control of glycogen metabolism [3]. The mechanism of activation of the ATP-Mg-dependent phosphatase has now been studied using different nucleotides and metal ions.

2. Materials and methods

Materials and methods were as described in [1]. The ATP-Mg-dependent phosphatase was commonly measured after a 10 min preincubation at 30°C of 1 μg/ml F_C with or without nucleotide, 0.5 mM metal chloride and 3 μg/ml F_A, as indicated. The activity (V) produced by 0.1 mM ATP (optimal concentration using ATP as nucleotide) was arbitrarily taken as 100%. One unit of phosphatase activity releases 1 nM [^{32}P]-phosphate/min at 30°C from ^{32}P-labelled phosphorylase *a* (2 mg/ml). All nucleotides were purchased from PL Biochemicals (USA), TPCK-treated trypsin from Worthington Biochemicals (England) and soybean trypsin inhibitor from Sigma Chemicals (USA).

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3. Results and discussion

3.1. *Nucleotide specificity*

Purified F_C (10 000 U/mg) was activated by F_A [2] (5000 U/mg), 0.5 mM $MgCl_2$ and different concentrations of nucleotides. As could be expected from [1,7], several nucleotides could efficiently replace ATP in the activation of the phosphatase (table 1). Adeno-

Table 1
Nucleotide specificity of the F_A-mediated activation of F_C in the presence of 0.5 mM Mg^{2+}

Nucleotide	*V* (%)	K_a (μM)
ATP	100	3
2'-Deoxyadenosine 5'-triphosphate	100	0.4
Adenosine 5'-tetraphosphate	70	10
α, β-Methyleneadenosine 5'-triphosphate	50	40
β, γ-Methyleneadenosine 5'-triphosphate	0	–
5'-Adenylylimidodiphosphate	0	–
Adenosine-5'-0-(3-thio)triphosphate	0	–
GTP	60–100	20
2'-Deoxyguanosine 5'-triphosphate	80–100	20
UTP	80	30
CTP	40	50
2'-Deoxycytidine 5'-triphosphate	30	300
ITP	40	500
Cyclic nucleotides: cAMP, cGMP, cCMP, cUMP or cTMP	no effect	–

Conditions are as indicated in the text

sine 5′-(*O-thio*)triphosphate was not effective in activating the phosphatase, although it could be used by F_A, which also has synthase kinase activity [2], to inactivate synthase *a* (not shown). The cyclic nucleotides tested could not replace ATP, and had no additional effect on the activation of the phosphatase produced by F_A and ATP-Mg.

3.2. *Metal ion specificity*

The effect of metal ions (0.5 mM metal chloride in all cases) on the activation of F_C was measured in 3 different ways. It was determined whether the metal ion alone could activate the phosphatase and what the effect was of added F_A alone, or in combination with 0.1 mM ATP. Maximal effects of all metal ions were obtained at 0.5 mM; higher doses of some of the metals were inhibitory (table 2). Mn^{2+} and Co^{2+} could replace Mg^{2+} in the F_A- and ATP-mediated activation of F_C with a >50% efficiency, but a distinctive difference between the former 2 metal ions and Mg^{2+} was that where Mg^{2+} alone does not cause any F_C-activation both Mn^{2+} and Co^{2+} can produce a substantial stimulation of the phosphatase activity without F_A and nucleotide present.

3.3. *Sensitivity of the ATP-Mg-dependent phosphatase to limited proteolysis by trypsin*

In [3] we stated that the inactive F_C-enzyme is very labile to trypsin, but becomes trypsin-resistant after activation by F_A and ATP-Mg. We have therefore compared the sensitivity to trypsin of the ATP-Mg-dependent phosphatase activities produced either by the activating metal ions alone or by the combination of F_A and ATP-metal. Metal ion was 0.5 mM in all cases, and F_A and/or 0.1 mM ATP was used where indicated. After an incubation with 0.2 mg/ml of TPCK-treated trypsin for 5 min at 30°C, proteolysis was stopped by the addition of 1 mg/ml of soy bean trypsin inhibitor. The results, shown in table 3 clearly indicate that no matter how the F_C-enzyme is activated, all of the phosphatase activity produced is resistant to limited proteolysis by trypsin. This would suggest that only before activation, the phosphatase is trypsin sensitive and that all activity produced from F_C, is trypsin resistant in analogy to the activity of the low molecular weight (M_r = 35 000) spontaneous phosphatases [8].

Table 2
Metal ion specificity of F_C-activation in the presence or absence of F_A and 0.1 mM ATP

Metal ion	% Phosphatase activity produced		
	Alone	+ (F_A)	+ (F_A + ATP)
Mg^{2+}	7	7	100
Mn^{2+}	35	30	55
Co^{2+}	40	28	60
Ni^{2+}	20	10	20
Zn^{2+}	–	–	–
Ca^{2+}	–	–	–

Conditions are as described in the text: metal ion concentration was 0.5 mM in all cases, and the phosphatase activity produced by F_A, 0.1 mM ATP and 0.5 mM Mg^{2+} was arbitrarily taken as 100%

Table 3
Effect of trypsin treatment on the activated F_C

Additions to F_C	% Phosphatase activity	
	Before trypsin	After trypsin
F_A	0	0
F_A + 0.1 mM ATP + 0.5 mM Mg^{2+}	100	100
0.5 mM Mg^{2+}	<5	<5
F_A + 0.1 mM ATP + 0.5 mM Mn^{2+}	55	50
0.5 mM Mn^{2+}	30	28
F_A + 0.1 mM ATP + 0.5 mM Co^{2+}	60	62
0.5 mM Co^{2+}	35	30

Conditions are specified in the text; phosphatase activities are expressed as % of the activity produced by F_A, 0.1 mM ATP and 0.5 mM Mg^{2+}

3.4. *Disproval of a phosphorylation as mechanism of activation of the ATP-Mg-dependent phosphatase*

The results in section 3.3 and in a [3] point to a metal transfer as possible mechanism of activation for F_C. Although we have reported in [1,9] that we were unable to detect any [^{32}P]phosphate incorporation into the F_C-proteins, it is always extremely difficult to provide conclusive evidence with negative data. We have therefore designed the following experiment. About 10 000 units of F_C (1 mg protein) were fully activated by F_A and [γ-^{32}P]ATP-Mg (spec. radioact. 1000 cpm/pM) and the activated enzyme preparation adsorbed onto a (1 × 1 cm)poly(Lys)–Sepharose 4B column equilibrated in a 20 mM Tris, 0.5 mM dithiothreitol (pH 7) buffer. F_A and ATP-Mg can be completely removed from the resin by a 0.2 M NaCl wash

and the phosphatase activity elutes with 0.5 M NaCl in the same buffer, as in [1,3]. The pooled fractions were concentrated by dialysis against 20 mM Tris, 0.5 mM dithiothreitol, 50% glycerol, pH 7, for 1 h on ice, after which 80% of the total phosphatase activity (measured after a preincubation with F_A and ATP-Mg) could still be attributed to activated F_C (measured without an additional preincubation with F_A and ATP-Mg): 20 μl of this contained 20 μg F_C proteins (or ~200 pmol F_C, assuming 80% purity of the F_C preparation, and based on a M_r = 70 000 for F_C [1]). Incorporation of 1 mol [^{32}P]phosphate/mol F_C would correspond to ~200 000 cpm of [^{32}P]phosphate covalently bound to the F_C proteins: 20 μl of this concentrated sample contained only 500 cpm of ^{32}P radioactivity. Two times 20 μl were directly treated with 20 μl sodium dodecyl sulfate buffer, and separately electrophoresed on 6% polyacrylamide gels in the presence of the detergent, as in [1]. One gel was stained for proteins using Coomassie brilliant blue (fig.1B) and consequently sliced into 2 mm portions; the other was sliced directly without staining. No significant ^{32}P radioactivity could be detected in any one of the slices from either gel (background cpm was ~50 and no slice contained >100 cpm). At the same time of the electrophoresis, 20 μl of the concentrated fractions were diluted 200-fold and incubated at 30°C. At regular time intervals, we measured the total phosphatase activity (+F_A, +ATP-Mg) and the 'activated F_C' activity (without F_A or ATP-Mg). Results are depicted in fig.1A and they clearly show that a time-dependent inactivation (or reversal of the F_C-activation) was taking place, although no [^{32}P]phosphate was present in the F_C proteins. These results were taken as absolute proof that the 'activated F_C' is not produced by a phosphate incorporation.

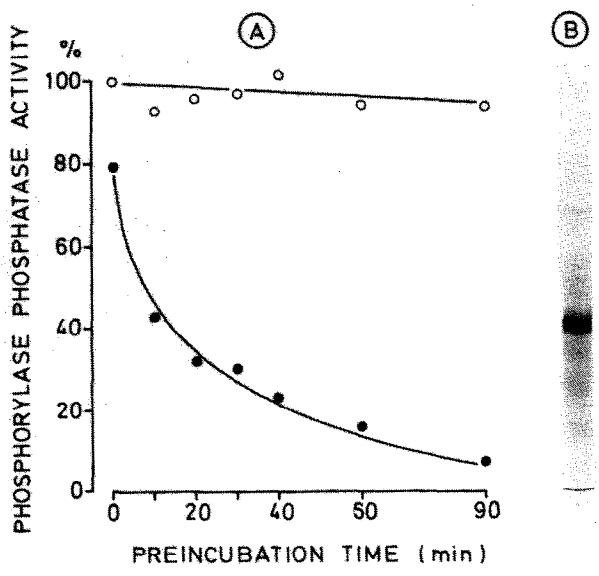

Fig.1. (A) Time-dependent reversal of the F_C-activation after poly(Lys)–Sepharose 4B chromatography by preincubation at 30°C. At the various time points, the preparation was assayed for total phosphatase activity (○) or for activated F_C (●). (B) Sodium dodecyl sulfate-gel electrophoresis of the activated F_C after poly(Lys)–Sepharose 4B chromatography.

4. Conclusions

A variety of nucleotides can replace ATP in the activation of the ATP-Mg-dependent phosphatase: not only do Co^{2+} or Mn^{2+} substitute for Mg^{2+} as ATP-metal donor, but these metal ions by themselves are able to produce considerable phosphatase activity from the inactive F_C-enzyme. The phosphatase activity created in all cases was trypsin insensitive, suggesting that proteolysis only destroys the inactive F_C enzyme. We had reported [3] that limited proteolysis of activated-F_C produces an irreversibly active phosphatase species, which no longer reverts back to the inactive F_C enzyme.

The fact that F_A can use adenosine 5′-(*O-thio*)-triphosphate to phosphorylate synthase *a*, but cannot use the nucleotide to activate F_C, suggest that the activation of the ATP-Mg-dependent phosphatase is not a phosphate incorporation. These data show that no [^{32}P]phosphate was incorporated into the F_C-proteins during the activation. Together with the multitude of reports in the literature [3,4,10–15] on metal ion dependency of various protein phosphatases, these results provide a plausible hypothesis for the molecular mechanism of activation of the ATP-Mg-dependent protein phosphatase:

It could involve the transfer of Mg ions from an ATP-Mg complex by the activating protein F_A. It is possible that this activation can be partially mimicked by certain metal ions (such as Co^{2+} and Mn^{2+}) alone. The variable inhibition of the phosphatase by metal chelating agents (such as EDTA, EGTA, fluoride, pyrophosphate and nucleotides) could be explained as a metal removal and conversion to an inactive form of phosphatase [3,11–21].

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