ACTIVATION OF POLYNUCLEOTIDE PHOSPHORYLASE BY 3′,5′-CYCLIC AMP, ATP-DEPENDENT PROTEIN KINASE

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1. Introduction

Protein kinase was first discovered in rabbit skeletal muscle [1] and was assigned the function of activating phosphorylase *b* kinase which in turn activates phosphorylase *b* [1, 2]. Other protein kinases have since been isolated from various sources [2–5]. Indeed this enzyme appears to be widely distributed in eucaryotic cells [6] and in *E. coli* [7]. The 3′,5′-cyclic AMP (cAMP)-dependent phosphorylation of protein also seems to exert a control over several enzymes in animal tissues, such as glycogen synthetase [8] and lipase [9]. However, little is known on its effect on bacterial enzymes, apart from phosphorylation of the σ factor of *E. coli* RNA polymerase [10]. The present communication deals with the stimulating effects of rabbit muscle protein kinase on polynucleotide phosphorylases.

2. Materials and methods

Rabbit skeletal muscle protein kinase was prepared according to the initial procedure described by Walsh et al. [1], up to the second DEAE-cellulose chromatography step. The A_{280}/A_{260} ratio was 1.70. The specific activity of the enzyme, measured by ^{32}P incorporation into histone with γ-^{32}P-ATP in the presence of cAMP, was 78,000 (pmoles ^{32}P incorporated/mg protein/5 min at 30°). The stimulation of this phosphorylation by cAMP was more than 10-fold.

E. coli polynucleotide phosphorylase was prepared according to Williams and Grunberg-Manago [11] with an additional sucrose gradient centrifugation step. The specific activity was 500 in the fresh preparation (defined as μmoles ADP released/mg protein/hr by phosphorolysis of poly A at 37° and pH 8).

The 'phosphorylating system' consisted of (mM): glycerophosphate buffer (pH 6.5) 10; $MgCl_2$ 10; EDTA 1; ATP 1; cAMP 0.01; and protein kinase in varying amounts.

PNP activity was mainly tested by incorporation of ^{14}C-ADP or ^{14}C-UDP into acido-insoluble polymers by the standard method [12]. The polymerization mixture contained (mM): tris (pH 8) 20; ^{14}C-ADP (or UDP) 5; $MgCl_2$, 2.5; and PNP 2–2.5 μg/ml.

The effect of protein kinase on PNP was assayed either by direct addition of 100 μl phosphorylating system (with 3–6 μg protein kinase) into 500 μl of polymerization mixture, or by preincubation of the enzyme with the phosphorylating system (4–5 μg PNP were incubated in 200 μl of phosphorylating system with 3–6 μg protein kinase at 37° for 10 min) followed by the dilution of this medium with the polymerization mixture.

3. Results

As shown in fig. 1, phosphorylation of *E. coli* polynucleotide phosphorylase (PNP) by protein kinase enhanced the activity of the former. The initial polymerization rate was increased 400–500%

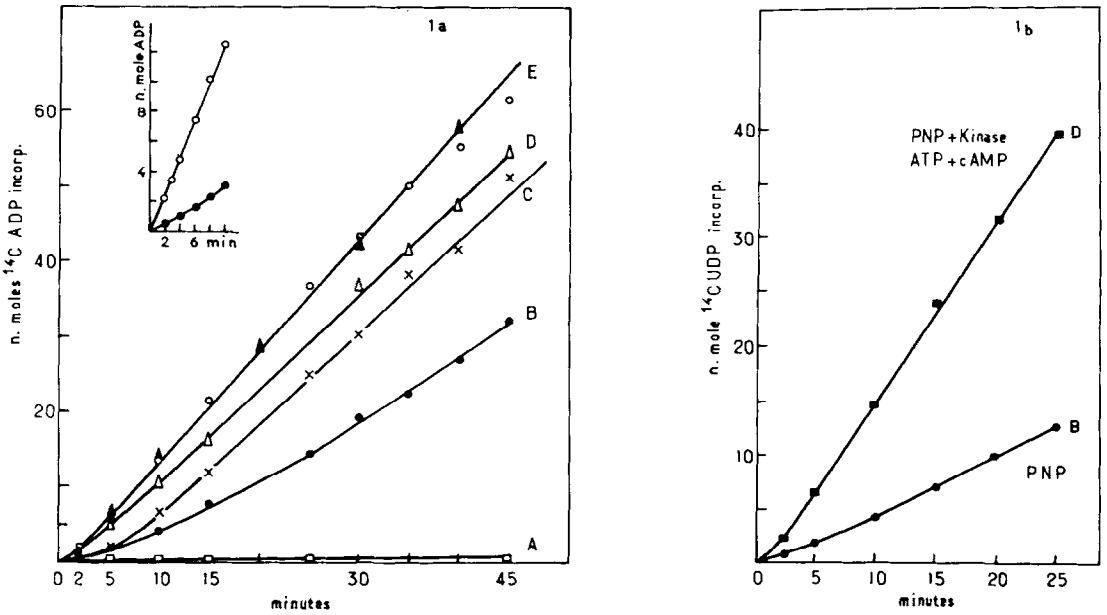

Fig. 1. Effect of protein kinase on polymerization by *E. coli* PNP of ^{14}C-ADP or ^{14}C-UDP. The conditions for the polymerization activity assay are described under Methods. All the incubation mixtures contained 2.1 μg/ml PNP, except for *curve A* where the mixture contained 12 μg/ml protein kinase alone. At indicated times, 100 μl aliquots were removed for determination of polymer formed. *Curve B*: PNP activity control. The same curve was obtained with: PNP alone; or in the presence of 2×10^{-6} M cAMP + 2×10^{-4} M ATP; or in the presence of cAMP + ATP + heated inactivated protein kinase (12 μg/ml); *curve C*: PNP + protein kinase (12 μg/ml); *curve D*: PNP + protein kinase (6 μg/ml) + cAMP (2×10^{-6} M) + ATP (2×10^{-4} M); *curve E*: o—o PNP + protein kinase (12 μg/ml) + cAMP (2×10^{-6} M) + ATP (2×10^{-4} M), ▲—▲ PNP + $(Ap)_3A$ (7×10^{-5} M).
Insert: PNP (2.1 μg) was first incubated in 100 μl of the phosphorylating system (with 6 μg protein kinase) at 37° C for 10 min, then diluted with the polymerizing mixture to the final conditions described for curve D. Control of preincubated PNP (o—o) was made under the same conditions, but in the absence of protein kinase.

for either ADP (fig. 1a, curve B, E) or UDP (fig. 1b). The stimulating effect was more significant when PNP was preincubated with the phosphorylating system. Indeed, not only did a prior incubation enhance PNP activity, but the lag phase observed with the untreated enzyme, was suppressed (fig. 1, insert).

It is known that the lag phase of polymerization can be overcome by the presence of oligonucleotides. Controls were therefore made to ascertain that the stimulation and the suppression of the lag phase were not due to either ATP or cAMP, nor to contaminants in these materials or in the kinase preparation. The presence of ATP and cAMP, alone, together, or mixed with inactivated kinase (heated 3 min at 90° in capillary tube) did not show any effect on PNP activity (fig. 1a, curve B). The most conclusive experiment eliminating the possibility of contamination is illustrated in fig. 2. When protein kinase was inactivated by repeated freezing and thawing, no stimulation of PNP activity was detectable under the conditions described in fig. 1; however, 50% of the stimulating effect could be recovered by increasing the concentration of cAMP and ATP.

It has been suggested that the attachment of an oligonucleotide primer is essential for the activity of PNP [13]. It is therefore remarkable that phosphorylation of the enzyme can be submitted for the role of the primer. Moreover, under optimal conditions, the extent of stimulation with an oligonucleotide primer is identical to that obtained by phosphorylation (fig. 1a, curve E). The simultaneous presence of oligomer and phosphorylating system did not give any additional effect.

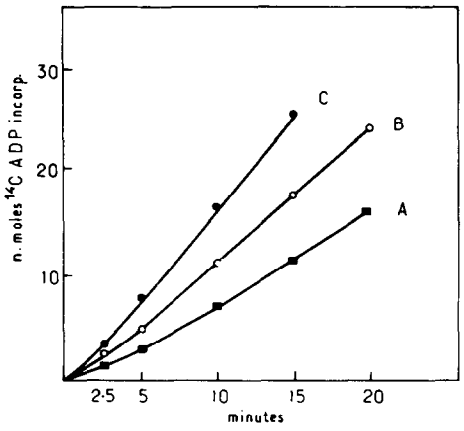

Fig. 2. Effect of cold inactivated protein kinase and dependence on cAMP and ATP concentrations. Protein kinase was inactivated by repeated freezing and thawing. PNP (1.3 μg) was preincubated 10 min at 37° in a volume of 50 μl containing either 9 μg cold inactivated protein kinase, 1.5×10^{-5} M ATP, and 0.8×10^{-5} M cAMP (*curve A*), or 4.5 μg cold inactivated protein kinase, 6×10^{-5} M cAMP (*curve B*). Control (*curve C*) contained 1.3 μg PNP in 50 μl glycerophosphate buffer incubated also at 37° for 10 min, and followed by the addition of 7×10^{-5} M $(Ap)_3A$. All three mixtures were diluted with 500 μl ^{14}C-ADP containing polymerizing mixture, and further incubated at 37°. At indicated times, 100 μl aliquots were removed for determination of polymers formed.

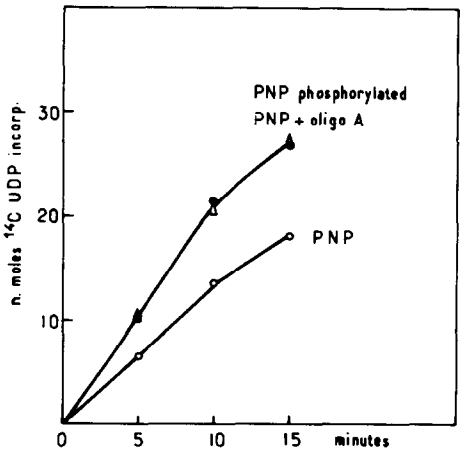

Fig. 3. Effect of protein kinase on *M. luteus* PNP. PNP isolated from *M. luteus* was purified according to [15] without the Zn-Sephadex step. PNP (50 μg/200 μl) was preincubated 10 min at 37° in the phosphorylating system with 6 μg protein kinase. Two other samples were incubated under the same conditions but without protein kinase. To one of these last samples 1×10^{-4} M $(Ap)_3A$ were added after the preincubation. All three samples were diluted with ^{14}C-UDP containing polymerizing mixture to a final volume of 600 μl. At indicated times, 100 μl aliquots were removed for determination of polymers formed.

With protein kinase alone (in the glycerolphosphate-Mg^{2+} buffer and in the absence of exogenous cAMP and ATP), we obtained a stimulating effect on PNP activity, though a short lag phase was observed (fig. 1a, curve C). This lag phase could be shortened on suppressed by preincubation of PNP with protein kinase in the presence of ATP (curve not shown). Muscle protein kinase has been shown to be highly dependent on cAMP; however, the cAMP-induced stimulation of the phosphorylating activity varies with the protein substrate. The enhancement of PNP activity observed in the presence of protein kinase alone might be due to the presence of ATP and cAMP already bound to the kinase. The ATP actually bound to the kinase could supply to some extent the nucleotide required for the phosphorylation. Indeed, the γ-^{32}P-ATP incorporation into PNP was very low in our experiments. It was estimated at 0.5 to 1 pmole Pi incorporated per pmole of PNP, assuming a molecular weight of 200,000 and 100% active molecules; this estimation is only semi-quantitative.

Phosphorylation of PNP did not markedly change the Michaelis constant for the substrate. In fact, K_m values for free ADP, calculated according to Williams et al. [14], were about 0.8 mM for untreated PNP, and 1 mM for phosphorylated PNP; these values are quite similar to that usually observed with *E. coli* PNP.

The phosphorolysis reaction catalyzed by PNP was also stimulated by protein kinase treatment. However, the degree of stimulation varied from one experiment to another. The reason for this variation is under investigation.

Another PNP, isolated from *M. luteus* was also tested. This enzyme is practically primer-independent: it is stimulated by oligonucleotide only to an extent of 30%. The treatment of this enzyme with protein kinase likewise produced a 30% stimulation (fig. 3).

4. Discussion

We know that cAMP is involved in controlling catabolic repression [16–19]. This is however not the case for the stimulation of the transcription of phage T4 DNA by *E. coli* RNA polymerase [10].

Polynucleotide phosphorylase is an enzyme involved in nucleic acid metabolism, albeit its ill-defined function in vivo, and is generally thought to be involved in the degradation of messenger or other RNA. This is why the observation of a stimulating effect by protein kinase on PNP is significant. The effect suggests that PNP could, in some way, be regulated by a mediator such as cAMP through phosphorylation. However, since we used an animal protein kinase, we are conscious of the limitation of our finding, although the presence of cAMP [20], of a cAMP-dependent protein kinase [7], and of a cAMP receptor protein [21] have been reported in *E. coli*.

Whether or not the phosphorylation is involved in the in vivo regulation of PNP, the fact that this enzyme can be phosphorylated, even with an animal system, opens a new possibility for the study of its mechanism of reaction and of its structure. It is striking that phosphorylation can replace the action of oligonucleotides for the suppression of the lag phase in the polymerization.

A model has been postulated recently, based on kinetics studies, in which the passage from inactive to active PNP involves the attachment of an oligo- or polynucleotide to the enzyme [13]. The data presented here enable us to postulate another model in which the control is mediated by the phosphorylation or dephosphorylation of the enzyme. The lag phase observed with purified PNP might thus result from partial or total loss of the Pi group.

Finally, the fact that there is a formal analogy between PNP activation which requires phosphorylation by a kinase or the presence of an oligonucleotide, and muscle phosphorylase *b* activation which also requires phosphorylation, or the presence of AMP, might constitute an interesting line of investigation.

Acknowledgement

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