The effect of fructose 2,6-bisphosphate and AMP on the activity of phosphorylated and unphosphorylated fructose-1,6-bisphosphatase from rat liver

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Rat liver fructose-1,6-bisphosphatase was partially phosphorylated in vitro and separated into unphosphorylated and fully phosphorylated enzyme. The effects of fructose 2,6-bisphosphate and AMP on these two enzyme forms were examined. Unphosphorylated fructose-1,6-bisphosphatase was more easily inhibited by both effectors. Fructose 2,6-bisphosphate affected both $K_{0.5}$ and V_{max}, while the main effect of AMP was to lower V_{max}. Fructose 2,6-bisphosphate and AMP together acted synergistically to decrease the activity of fructose-1,6-bisphosphatase, and since unphosphorylated and phosphorylated enzyme forms are affected differently, this might be a way to amplify the effect of phosphorylation.

Fructose-1,6-bisphosphatase *Fructose 2,6-bisphosphate* *AMP* *Chromatofocusing* *Regulatory phosphorylation* *Cyclic AMP-dependent protein kinase*

1. INTRODUCTION

The newly discovered sugar diphosphate, fructose 2,6-bisphosphate ($F2,6P_2$), has become of increasing interest during the last few years. Its effect on 6-phosphofructo-1-kinase has been thoroughly investigated, and it has been reported that phosphorylation of this enzyme decreases its affinity for the effector [1]. The most striking influence of $F2,6P_2$ on this enzyme is to decrease the inhibitory effect of ATP [1].

The bifunctional enzyme responsible for the synthesis and degradation of $F2,6P_2$ has been purified [2], and the action of various effectors on its activity has been investigated [3,4]. In addition, studies of the hormonal regulation of the enzyme show that the level of $F2,6P_2$ can be lowered by both glucagon and epinephrine [5,6].

It has been reported by several authors that fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11; $F1,6P_2$ase) is inhibited by $F2,6P_2$. This has been shown for $F1,6P_2$ase in organs from various mammals such as rabbit liver [7], rabbit muscle [8], swine kidney [9,10], rat liver [8,11,12] and bovine liver [13]. The nature of this inhibition has attracted much interest, especially when it was found to be synergistic with the effect of AMP [7–11], a well known allosteric inhibitor of $F1,6P_2$ase. $F2,6P_2$ is currently believed to be a purely competitive inhibitor, but a recent suggestion is that it also interacts with a site other than the catalytic one [13].

Rat liver $F1,6P_2$ase is known to be phosphorylated in vitro by cAMP-dependent protein kinase, leading to a decreased $K_{0.5}$ for $F1,6P_2$ [14]. Reports in the literature concerned with the process in vivo have been inconclusive on this point: authors in [14] reported an increase in activity of the enzyme whereas those in [15] found no change in the activity of phosphorylated $F1,6P_2$ase.

Here we have separated phosphorylated and un-

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phosphorylated F1,6P$_2$ase. This has not been done previously, perhaps due to the difficulty encountered in obtaining completely phosphorylated enzyme, particularly as the phosphate-receptor sequence is prone to proteolytic cleavage [16,17].

When the activities of unphosphorylated and phosphorylated F1,6P$_2$ase were compared in the presence of F2,6P$_2$ and/or AMP it was observed that the unphosphorylated form of F1,6P$_2$ase was more sensitive to inhibition by both effectors.

2. MATERIALS AND METHODS

2.1. *Materials*

Bovine serum albumin, AMP cyclic AMP, dithiothreitol (DTT), NADP, F1,6P$_2$, F2,6P$_2$ and Coomassie brilliant blue G-250 were from Sigma. [^{32}P]ATP was bought from New England Nuclear. Sephadex G-50, Polybuffer Exchanger PBE 94 and Polybuffer 74 were purchased from Pharmacia, Uppsala. Glucose-6-phosphate dehydrogenase and phosphohexose isomerase were from Boehringer. All other reagents used were of the highest degree commercially available.

2.2. *Methods*

2.2.1. Preparation of F1,6P$_2$ase

F1,6P$_2$ase was purified from rat liver as in [14]. This yielded an enzyme which was homogeneous as judged from polyacrylamide gel electrophoresis in detergent, and with an apparent subunit M_r of 37 000 [18]. The specific activity was 9–14 units/mg and the ratio of activity at pH 7.5 to that at pH 9.2 was 1.6 for the purified enzyme and did not change after chromatofocusing. This ratio is below 1 for enzyme modified with subtilisin as reported in [19].

2.2.2. Purification of protein kinase

The catalytic subunit of cAMP-stimulated protein kinase was prepared as in [20], but with the modifications in [18]. One unit is defined as the amount of enzyme that can transfer 1 pmol phosphate from ATP to mixed histone per min at 30°C.

2.2.3. Phosphorylation of F1,6P$_2$ase

Purified rat liver enzyme (430 μg) was phosphorylated with 420 units of catalytic subunit from cAMP-dependent protein kinase and 0.5 mM [^{32}P]ATP as in [18]. It was possible to phosphorylate the enzyme to 1 mol/mol enzyme subunit, but to obtain similarly treated samples of phosphorylated and unphosphorylated enzyme the reaction was interrupted earlier. After 1 h at 30°C in the presence of 25 mM 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.9), 4 mM magnesium acetate, 2 μM cAMP, 1 mM DTT, the reaction was terminated by chromatography on a Sephadex G-50 column equilibrated with 25 mM histidine–HCl buffer (pH 7.0), 1 mM DTT, 0.1 mM F1,6P$_2$, 30% glycerol. The protein was eluted in the void volume.

2.2.4. Chromatofocusing of F1,6P$_2$ase

The phosphorylated enzyme was chromatofocused on a column (0.6 × 3.5 cm) packed with Polybuffer Exchanger, equilibrated with 25 mM histidine-HCl buffer (pH 5.75), 1 mM DTT, 0.1 mM F1,6P$_2$, 30% glycerol. Three mg protein was applied per ml gel. Prior to application the pH of the sample was adjusted to 5.75.

The F1,6P$_2$ase was eluted with Polybuffer 74 (diluted 10 times) (pH 3.5), 1 mM DTT, 0.1 mM F1,6P$_2$, 30% glycerol. Fractions of 200 μl were collected in test tubes to which 50 μl 1 M Tris–HCl buffer (pH 7.5) had been added to minimize exposure of the enzyme to low pH. Some tubes left without these additions were used for pH determination, using a microelectrode unit (Radiometer, Copenhagen).

2.2.5. Dephosphorylation of F1,6P$_2$ase

Phosphorylated and chromatofocused F1,6P$_2$ase (0.57 nmol subunit) was treated with 0.13 unit of low M_r phosphoprotein phosphatase, prepared as in [21]. One unit of phosphatase is the amount of enzyme which can release 1 nmol [^{32}P]-phosphate from [^{32}P]phosphohistone per min at 30°C. The reaction took place in the presence of 50 mM Tris–HCl buffer (pH 7.5), 6 mM DTT, 1.25 mM $MgSO_4$, 0.1 mg/ml bovine serum albumin at 30°C in a total volume of 2 ml. The material was used for activity measurements.

2.2.6. Assay of F1,6P$_2$ase

The analyses were performed essentially as in [18]. The amount of sample used in the test was chosen to give an activity of 4.5 milliunits, when measured at optimal substrate concentration. One unit is defined as the amount of enzyme that can dephosphorylate 1 μmol F1,6P$_2$ per min. The purified enzyme had a specific activity of 10 units/mg protein as reported in [18].

The reaction mixture consisted of 25 mM potassium phosphate buffer (pH 7.5), 25 mM 2-

mercaptoethanol, 1 mg/ml bovine serum albumin, 2.5 mM $MgSO_4$, 0.4 mM NADP, 0.7 unit glucose-6-phosphate dehydrogenase, 0.35 unit phosphohexose isomerase; final volume 600 μl. This mixture was preincubated with the sample (5–40 μl, determined as above) for 3 min at 30°C. Prior to the kinetic studies, the enzyme was dialyzed against 25 mM histidine-HCl buffer (pH 7.0), 1 mM DTT, 30% glycerol.

In analyses where F2,6P_2, AMP or both were present, the effectors were preincubated together with the enzyme. F2,6P_2 was dissolved in 0.5 mM Tris-HCl (pH 8.6). To ensure that the noted effect was independent of pH, an equal amount of this buffer was added to the samples, which were assayed without the sugar derivative.

The reaction was started by the addition of F1,6P_2, giving final concentrations up to 33 μM, and the reaction took place at 30°C.

2.2.7. *Other methods*

To check whether the radioactivity found in the chromatograms was bound to proteins, aliquots containing between 2000 and 10 000 cpm (Čerenkov radiation) were taken, 1 ml bovine serum albumin was added, and the protein was precipitated with 2 ml ice-cold trichloroacetic acid (10%, w/v) with 50 mM phosphoric acid and the radioactivity in the pellet, dissolved in 0.5 ml of 0.5 M NaOH, was measured. All protein determinations were made as in [22].

3. RESULTS AND DISCUSSION

Chromatofocusing of partially phosphorylated F1,6P_2ase gave two activity peaks – one with an apparent p*I* of about 4.5, the other of about 5.0 (fig.1). The yield of activity was 95–100% in the chromatofocusing step and the specific activity was still 10 units/mg. Reports on the specific activity of F1,6P_2ase vary between 6 [23] and 45 units/mg [17]. Since the concentrations of effectors vary in the assays described by different authors, it is difficult to make a direct comparison of the values. In this laboratory values of 9–14 units/mg for a homogeneous preparation are typical. Some comments on the influence of dif-

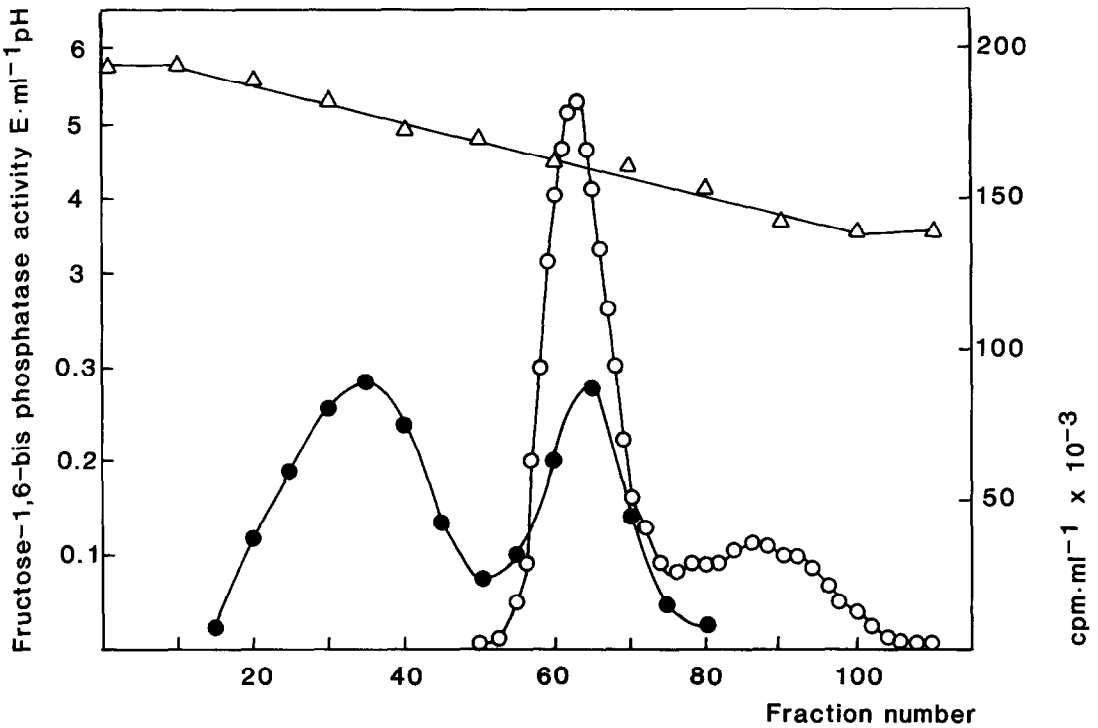

Fig. 1. Chromatofocusing of F1,6P_2ase, partially phosphorylated in vitro. Enzyme activity at 80 μM F1,6P_2 (●—●), cpm/ml (○—○) and pH gradient (△—△) were measured.

ferent effectors on the activity of F1,6P$_2$ase can be found in [24].

Most of the radioactivity was recovered in the peak with lower p*I*. Following this peak, and well separated from it, another peak with a smaller amount of radioactivity appeared. It was found that more than 99.9% of the radioactivity in the peak with p*I* 4.5 was protein-bound. This value was less than 0.5% for the latter. The non-protein-bound radioactivity represents ATP, which was adsorbed to the proteins, and therefore was not fully separated from the enzyme under the conditions used for chromatography on the Sephadex G-50 column.

The F1,6P$_2$ase found at pH 5.0 was completely unphosphorylated and displayed a $K_{0.5}$ of 15 μM for F1,6P$_2$ and the enzyme form with lower p*I* was completely phosphorylated and showed a $K_{0.5}$ of 10 μM. This should be compared with the values reported earlier, where it has been found that phosphorylation in vitro decreased $K_{0.5}$ from 22 to 11 μM [18]. After dephosphorylation of the phosphorylated chromatofocused F1,6P$_2$ase the $K_{0.5}$ coincided exactly with that of unphosphorylated enzyme prior to chromatofocusing. The unphosphorylated enzyme was subjected to the same additions except for the phosphoprotein phosphatase.

It was demonstrated that F,6P$_2$ase, fully phosphorylated in vitro, was less sensitive to inhibition by F2,6P$_2$ than the unphosphorylated form of the enzyme. As seen in fig.2 the concentration required for 50% inhibition, measured at 12.5 μM F1,6P$_2$, was found to be 4 μM for the phosphoenzyme and 2 μM for the unphosphorylated form. At higher substrate concentration (33 μM) the corresponding values were found to be 11 and 4 μM, respectively.

The amount of F2,6P$_2$ needed for 50% inhibition of the unphosphorylated enzyme reported here is in good agreement with values reported for unphosphorylated F1,6P$_2$ from rat liver (3.5 μM [8], 1.4 μM [11]), swine kidney (2.5 μM [9], 2 μM [12]) and rabbit liver (5 μM [7]).

The results suggested that the inhibitor acts in a competitive fashion for both enzyme forms, and the K_i values were estimated to be about 1.5 μM for the unphosphorylated and 3 μM for the phosphorylated enzyme.

It was reported in [12] that F1,6P$_2$ase in cytosol from rats injected with glucagon is activated, probably due to phosphorylation of the enzyme in response to the hormone. At the same time, the sensitivity of the enzyme to inactivation by F2,6P$_2$ is lowered so that the K_i value of 0.1 μM observed for control animals is approximately doubled on injection of glucagon. These results could not be expected to coincide exactly with ours since we work with an in vitro system with purified enzymes, and these authors with liver cytosol, containing all metabolites of the cell.

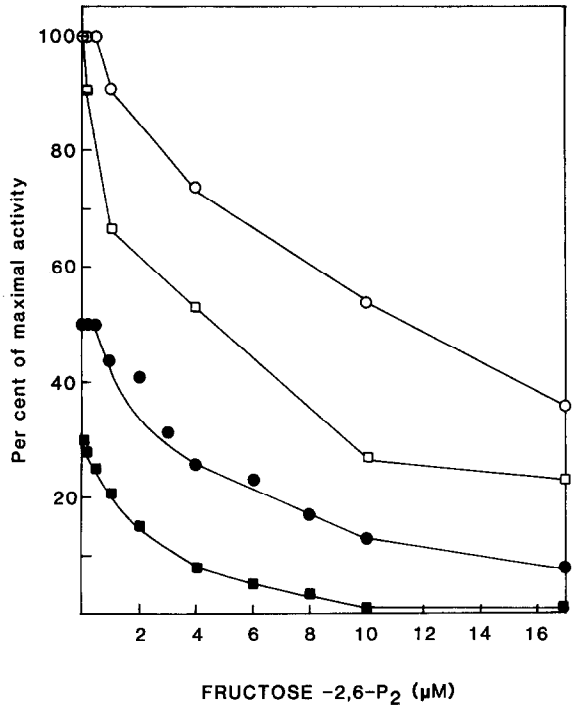

Fig. 2. Inhibition of rat liver F1,6P$_2$ase by F2,6P$_2$. Activity of phosphorylated and unphosphorylated enzyme was measured at 33 and 12.5 μM F1,6P$_2$. The amount of inhibitor was varied as indicated. Phosphorylated enzyme measured at 33 μM (○—○) and 12.5 μM (●—●) and unphosphorylated enzyme at 33 μM (□—□) and 12.5 μM (■—■) substrate.

Authors in [11] reported a K_i of 0.5 μM when working with purified unphosphorylated F1,6P$_2$ase from rat liver. One possible explanation for the discrepancy could be differences in Mg^{2+} concentration. Authors in [25] showed that Mg^{2+} inhibits the binding of F2,6P$_2$ to F1,6P$_2$ase: at 250 μM Mg^{2+} only 0.28 mol F2,6P$_2$ had bound per mol subunit compared to 0.50 mol/mol at 50 μM and 0.61 mol/mol in the absence of Mg^{2+}. The concen-

trations used were 2 mM plus 0.1 mM EDTA [11] and 2.5 mM (here). These concentrations are well within the range of physiological concentration of 5–10 μmol/g wet wt reported in [26].

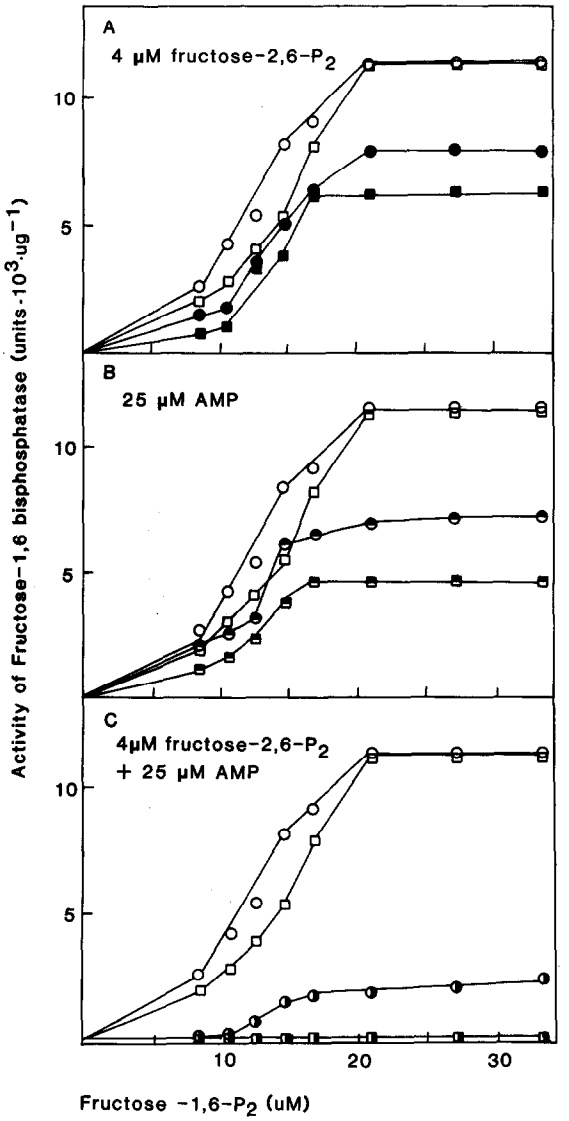

Fig. 3. The influence of $F2,6P_2$ and AMP on the activity of $F1,6P_2$ase. Activity of phosphorylated (○—○) and unphosphorylated (□—□) enzyme without inhibitor. (A) Phosphorylated (●—●) and unphosphorylated (■—■) $F1,6P_2$ase assayed in the presence of 4 μM $F2,6P_2$. (B) Phosphorylated (◐—◐) and unphosphorylated (◨—◨) enzyme with 25 μM AMP. (C) Phosphorylated (◑—◑) and unphosphorylated (◧—◧) enzyme with both 4 μM $F2,6P_2$ and 25 μM AMP.

$F2,6P_2$ at 4 μM was also found to lower V_{max} for both enzyme forms to about 70% of the uninhibited value for phosphorylated and 55% for unphosphorylated and dephosphorylated $F1,6P_2$ase (fig.3A). The concentrations of $F1,6P_2$ used in these experiments were chosen to avoid the substrate inhibition observed at high concentrations in [8].

Addition of 25 μM AMP lowered V_{max} to 70% (phosphorylated) and 40% (unphosphorylated, fig.3B, and dephosphorylated forms, fig.4) of uninhibited values.

AMP plus 4 μM $F2,6P_2$ added simultaneously had a very dramatic effect. V_{max} was decreased to 20% for the phosphorylated enzyme form, and only 5% activity was found for the unphosphorylated one under our conditions (fig.3C). This latter inhibition is more than additive, and indeed, all authors working with mammalian unphosphorylated $F1,6P_2$ase have reported that AMP and $F2,6P_2$ act synergistically [7–11]. Authors in [25] have also shown that up to 20 μM AMP has no effect on the amount of $F2,6P_2$ bound to $F1,6P_2$ase, so it seems to be certain that the two inhibitors indeed bind to different sites in the molecule.

The most reasonable conclusion from our results is that the amount of $F2,6P_2$ bound to $F1,6P_2$ase varies with the state of phosphorylation of the enzyme, thus modifying its activity. There has been no comparative study of the binding capacity of

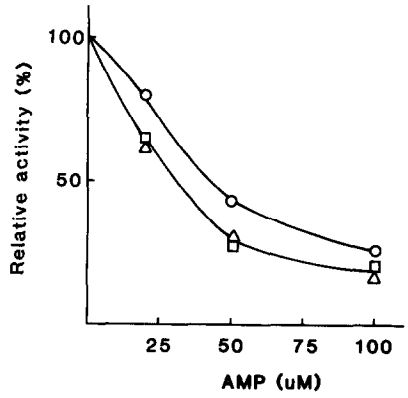

Fig. 4. The effect of AMP on the activity of $F1,6P_2$ase. Activity of phosphorylated (○—○), dephosphorylated (□—□) and unphosphorylated (△—△) $F1,6P_2$ase measured at 33 μM $F1,6P_2$. The concentration of AMP was varied as indicated.

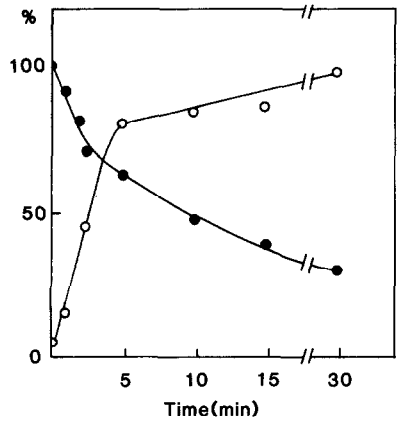

Fig. 5. Dephosphorylation of F1,6P$_2$ase. Change in activity (●—●) of phosphorylated F1,6P$_2$ase and release of [^{32}P]phosphate (○—○) during dephosphorylation. The reaction was performed as described in section 2 and samples were withdrawn and analysed at the times indicated. The enzymatic activity was measured at 20 μM F1,6P$_2$ in the presence of 4 μM F2,6P$_2$ and 25 μM AMP. The initial activity was 100% and all the radioactivity was released after 30 min incubation.

the two enzyme forms, but it seems probable that unphosphorylated enzyme binds both F2,6P$_2$ and AMP more tightly than does its phosphorylated counterpart. This is analogous to the results reported for 6-phosphofructo-1-kinase [27,28] where unphosphorylated enzyme is reported to show greater affinity for F2,6P$_2$ which, in this case, results in a decreased sensitivity to ATP inhibition.

Treatment of hepatocytes with glucagon induces phosphorylation of not only F1,6P$_2$ase and 6-phosphofructo-1-kinase but also of the bifunctional enzyme 6-phosphofructo-2-kinase/F2,6P$_2$-ase [2], which leads to inhibited synthesis and increased degradation of F2,6P$_2$ and a dramatic decrease in the concentration of the effector from about 16 to about 2 nmol/g [29]. So as a result of glucagon administration the amount of unphosphorylated F1,6P$_2$ase, more sensitive to inhibition by the effector, and that of unphosphorylated 6-phospho-1-kinase, more easily activated by the effector, are both lowered, as is the concentration of the effector itself. The total result will be stimulation of gluconeogenesis and inactivation of glycolysis, a well known consequence of glucagon administration or starvation.

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