Volume 261, number 1, 23–27 FEBS 08086 February 1990

The ammonium sulfate activation of phosphorylase *b*

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Received 17 December 1989

The ammonium sulfate activation of phosphorylase *b* has been studied. Ammonium sulfate, when present in high concentrations, induces properties of phosphorylase *a* in phosphorylase *b*, such as an enhanced affinity for AMP, a reversal of the glucose-6-P inhibition and enzyme tetramerization. The data are consistent with the interpretation that sulfates bind to the Ser-14 site and the sulfate-protein interactions at this site are responsible for activation of phosphorylase *b*.

Phosphorylase *b*; Phosphorylase *a*; Activation; Electron spin resonance; Tetramerization; Structure-function

1. INTRODUCTION

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the reversible transfer of a glucosyl group from the non-reducing end of an α-1,4-glucosidic polysaccharide to orthophosphate to form glucose-1-P. It is an allosteric enzyme that exists in two interconvertible (by phosphorylation and dephosphorylation of one specific serine residue) forms, *b* and *a* [1]. Phosphorylase *b* is inactive but can be allosterically activated by AMP or IMP and inhibited by glucose-6-P and ATP. Phosphorylase *a* is the active species but it can be further activated by AMP to some extent. Both forms of the enzyme are allosterically inhibited by caffeine and glucose. The AMP activation or the phosphorylation of phosphorylase *b* can be understood as a conversion from a dimeric T-state (low affinity) to a dimeric R-state (high affinity) while the action of the inhibitors can be understood in terms of stabilization of the T-state [2–4]. In vitro rabbit muscle phosphorylase undergoes a dimer to tetramer conversion upon activation, but binding of T-state inhibitors can lead to dissociation of the tetramers [2,5–7]. Most of our knowledge on the structural aspects of the T to R allosteric transition of the enzyme has been based on the high resolution X-ray analysis of the two forms of the enzyme in the T-state and their complexes with a number of substrates, substrate analogues and effectors (reviewed in [8]).

Preliminary studies have previously shown that ammonium sulfate, when present at high concentrations, is able to both induce an AMP-independent activity [9] and stimulate the AMP-dependent activity [10] of glycogen phosphorylase *b*. The growth of a monoclinic crystal of phosphorylase *b*, in the presence of 1.0–1.2 M of ammonium sulfate, which contains one tetramer per asymmetric unit has been reported a long time ago [11–13] but its three-dimensional structure was only recently described [14]. The X-ray analysis at a resolution of 2.9 Å revealed tertiary structural changes in response to sulfate binding. These changes, concomitant with conversion of the enzyme to the active R-state conformation, are coupled to large changes in quaternary structure which directly affect the AMP and the Ser-14 phosphate site and indirectly the catalytic site [14].

Comparison of results obtained with X-ray crystallographic techniques with those obtained by solution techniques should give a better understanding of the salt activation process of the enzyme. In this report, the effect of ammonium sulfate on the solution properties of phosphorylase was studied by kinetic, spectroscopic and ultracentrifugation experiments. The results show that ammonium sulfate has large effects on the kinetics and structure of the enzyme.

2. MATERIALS AND METHODS

Rabbit skeletal muscle phosphorylase *b* and phosphorylase *a* were prepared and recrystallized as described in [15]. Bound nucleotides were removed as in [16]. The enzyme concentration was determined from absorbance measurements at 280 nm [6] and calculations of phosphorylase *b* molarity were based on a molecular weight of 97434 for monomer *b* [8]. Phosphorylase activity was determined at pH 6.8

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Abbreviations: glucose-1-P, α-D-glucopyranose-1-phosphate; glucose-6-P, glucose-6-phosphate; 4-(2-iodoacetamido)2,2,6,6-tetramethyl piperidinyloxyl, iodoacetamide spin label; ESR, electron spin resonance; glycogen phosphorylase, 1,4-α-D-glucan; orthophosphate α-glucosyltransferase (EC 2.4.1.1)

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00145793/90/$3.50

and 30°C in the direction of glycogen synthesis by measuring the inorganic phosphate released in the reaction according to [17]. The enzymes (5–10 μg/ml) were assayed in 10 mM β-glycerophosphate, 0.5 mM EDTA and 1 mM dithiothreitol with 1% glycogen and various concentrations of glucose-1-P, AMP and ammonium sulfate as indicated. Enzyme and glycogen were preincubated for 15 min at 30°C before initiating the reaction with glucose-1-P. Preincubation of enzyme with glycogen in the presence of ammonium sulfate did not affect the kinetics. Initial velocities were calculated from the pseudo-first-order rate constants [18]. The K_ms for glycogen shown in table 1 were determined from initial velocities calculated according to [19] by using glycogen concentrations between 0.005 and 1%.

Modification of phosphorylase *b* by 4-(2-iodoacetamido)2,2,6,6-tetramethyl piperidinyloxyl (purchased from Sigma Chemical) was performed with a 10% excess of the spin label as in [20], except that AMP was not added in the reaction mixture. ESR spectra were recorded on a Bruker ER 200D spectrometer at room temperature (approx. 25°C) under conditions described in [21], and characterized by the ratio (ESR ratio) of the low field to the center peak heights in the differentiated form of the spectrum. The change in the ESR ratio was used to follow AMP binding [22,23].

For the calculation and statistical evaluation of the kinetic and binding parameters, a non-linear regression program was used [24].

Ultracentrifugation experiments were performed on a Beckman Model L5-50 analytical ultracentrifuge at a rotor speed of 50 000 rpm and 16 ± 1° and 20 ± 1°C. All sedimentation velocity measurements were performed with 10 mg/ml phosphorylase *b* in 14 mM β-glycerophosphate buffer (pH 6.9) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol. The movement of protein boundaries was followed by Schlieren optics. Pictures were taken at various times after the rotor had reached full speed. Sedimentation coefficients calculated by the sedimentation velocity method were corrected for viscosity and density of the buffer to water at 20°C [25].

Oyster glycogen (Serva) was freed of AMP according to [26]. AMP, glucose-1-P (dipotassium salt) and ammonium sulfate were products of Sigma Chemical and sodium β-glycerophosphate was obtained from E. Merck.

3. RESULTS

3.1. *Kinetics*

The extent of phosphorylase *b* activation, both by ammonium sulfate and AMP with respect to glucose-1-P at a constant concentration of glycogen (1%), is presented in fig.1 for the purpose of comparison. Ammonium sulfate activation is characterized by an increase in the V_{max} value with a significant change in the affinity for the substrate. The increase in K_m is probably due to an effect of ionic strength or to a partial competition between glucose-1-P and ammonium sulfate (see below). AMP-activated phosphorylase *b* shows an increase in the maximal activity and a decrease in the K_m for glucose-1-P, in agreement with previous results [27]. The activation pattern of phosphorylase *b* in the absence or presence of AMP with respect to glucose-1-P at constant concentrations of glycogen (1%) and ammonium sulfate (0.9 M) is presented in fig.2. In the absence of AMP, the enzyme showed a maximal activity of 29 ± 1 IU/mg but a decrease affinity for the substrate glucose-1-P. However, in the presence of ammonium sulfate, a dramatic stimulation of AMP-dependent activity of the enzyme was observed. Using the general scheme for non-essential activation [28], from the family of intersecting plots, dissocia-

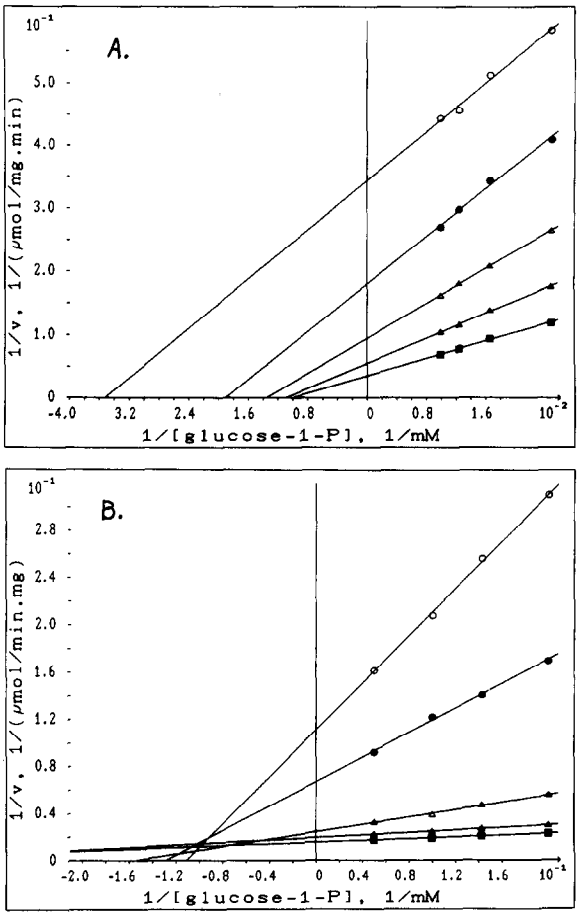

Fig.1. (A) Double-reciprocal plot of ammonium sulfate activation of phosphorylase *b* at varying concentrations of glucose-1-P constant concentration of glycogen (1%). Activator concentrations were: 0 (○), 0.2 (●), 0.4 (△), 0.6 (▲) and 0.8 (■). (B) As in (A) except that ammonium sulfate was substituted with AMP at concentrations of 10 (○), 20 (●), 40 (△), 100 (▲) and 1000 μM (■).

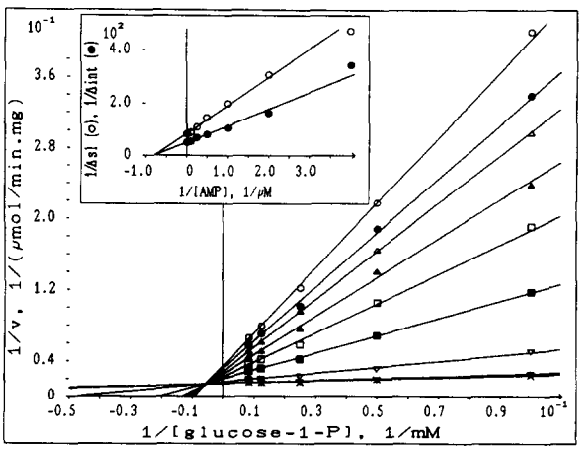

Fig.2. The activity of phosphorylase *b* as a function of glucose-1-P concentration at constant concentrations of glycogen (1%) and ammonium sulfate (0.9 M) and the following concentrations of AMP: 0 (○), 0.25 (●), 0.5 (△), 1.0 (▲), 2.0 (□), 4.0 (■), 10 (▽), 100 (▼) and 1000 μM (X). Assays were performed as described in section 2. Inset, secondary reciprocal plots of 1/Δ slope (multiplied by 300) and 1/Δ intercept vs1/[AMP]. Δ slope and Δ intercept values represent the control values (without AMP) minus the values obtained in the presence of AMP [28].

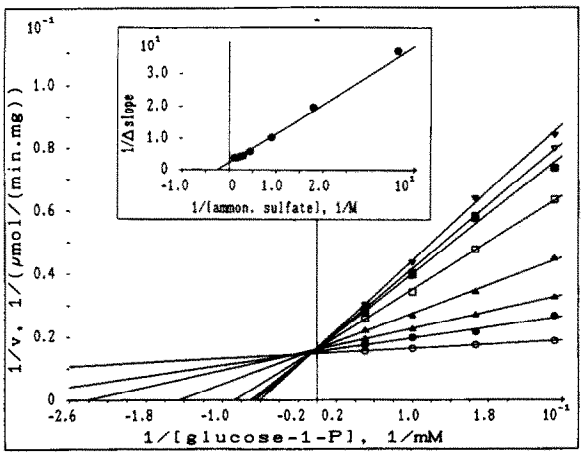

Fig.3. The activity of phosphorylase *a* as a function of glucose-1-P concentration at a constant concentration of glycogen (1%) and various concentrations of ammonium sulfate. Ammonium sulfate concentrations were as follows: (○) none, (●) 0.028, (△) 0.055, (▲) 0.11, (□) 0.22, (■) 0.33, (▽) 0.44 and (▼) 0.67 M. Inset, secondary plots of 1/Δ slope (as defined in fig.2) vs 1/[ammonium sulfate].

tion constants $K_s = 108 \pm 3$ and $\alpha K_s = 6.5 \pm 0.5$ mM were calculated for glucose-1-P binding to the enzyme-glycogen and enzyme-glycogen-AMP complexes, respectively, in the presence of ammonium sulfate. From the secondary replot of fig.2 (inset), the dissociation constants for AMP from the enzyme-glycogen-AMP (K_a) and the enzyme-glycogen-glucose-1-P-AMP (αK_a) complexes in the presence of ammonium sulfate were also obtained. They were 48.9 and 3.1 μM, respectively. The maximal activity obtained at 0.9 M ammonium sulfate and 1 mM AMP was 70 ± 1 IU/mg. Similar results were obtained when the effects of IMP on the ammonium sulfate activation of phosphorylase *b* were examined (data not shown). Dissociation constant values of 1.6 and 0.12 mM were observed for IMP binding to the enzyme-glycogen and enzyme-glycogen-glucose-1-P complexes in the presence of 0.9 M ammonium sulfate.

In contrast, phosphorylase *a* is inhibited rather than activated by ammonium sulfate. Cori et al. [29] in their classical paper have studied the effect of ammonium sulfate on the activity of the enzyme at one concentration of glucose-1-P (16 mM) and reported an inhibition constant of 26 mM for the salt effect. The effect of ammonium sulfate concentration on the activity of phosphorylase *a* as a function of glycose-1-P concentration is shown in fig.3. Since the velocity of the reaction cannot be reduced to zero as the salt concentration increase to infinity, an hyperbolic mixed-type inhibition is indicated [28]. To a first approximation, the plots can be treated according to the scheme for partial competitive inhibition from which inhibition constant (K_i) values of 25 and 390 mM can be calculated for salt binding to the enzyme-glycogen and enzyme-glycogen-glucose-1-P complexes, respectively.

A series of known inhibitors were tested for their effect on the activity of phosphorylase *b* (± 1 mM AMP), phosphorylase *a* (± 1 mM AMP) and phosphorylase *b* (± 1 mM AMP) in the presence of ammonium sulfate. The results are summarized in table 1.

3.2. *Ultracentrifugation*

The effect of ammonium sulfate on the sedimentation properties of phosphorylase *b* was investigated. In

Table 1

Kinetic constants of phosphorylase *b* and phosphorylase *a* in the absence and presence of 1 mM AMP and the effect of ammonium sulfate on the kinetic constants of phosphorylase *b*

	Phosphorylase *b*		Phosphorylase *a*		Phosphorylase *b* + 0.9 M ammonium sulfate	
	−AMP	+AMP	−AMP	+AMP	−AMP	+AMP
V_{max} glucose-1-P (IU/mg)	2.9± 0.1	64 ±1	67.2 ±0.1	74 ±1	29 ±1	70 ±1
K_m glucose-1-P (mM)	28 ± 2	9.0 ±2.4	1.07±0.01	0.30±0.05	108 ±3	6.5 ±0.5
K_m glycogen × 1000 (%)	47 ± 6 [a]	12 ±1 [b]	6.7 ±0.1[b]	4.7 ±0.4[b]	4.1 ±0.6[a]	1.3 ±0.1[b]
K_α AMP (μM)	63 ±49[c]	–	0.3 ±0.1[d]	–	3.1	–
K_α IMP (mM)	1.7± 3.8[e]	–	0.005[f]	–	0.12[k]	–
K_i glucose (mM)	–	3.2 ±0.2[g]	4.9 ±0.6[g]	21 ±4[g]	11 ±1[l]	32 ±2[m]
K_i caffeine (mM)	–	0.13±0.01[g]	0.17±0.02[g]	2.1 ±0.5[g]	0.039±0.004[n]	0.78±0.03[o]
K_i glucose-6-P (mM)	–	0.52±0.03[h]	1.7[i]	9.1 ±0.2[j]	10.6 ±0.7[p]	107 ±2[p]

Enzymes were assayed as described in section 2 and figs. 1–2. Glucose, caffeine and glucose-6-P tested at several concentrations acted as competitive inhibitors with respect to glucose-1-P. K_is were determined as in [33]

[a] Determined at 100 mM glucose-1-P. [b] Determined at 20 mM glucose-1-P. [c] Calculated from the data of fig.1B from Hill plots according to [2]. Hill coefficient was varied between 1.4 and 1.5 over a 5–20 mM glucose-1-P concentration range. [d] Determined at 20 mM glucose-1-P. [e] Calculated from the data of [34]. [f] Data from [35]. [g] Data from [15]. [h] Determined at 1, 2, 4 and 10 mM glucose-6-P. [i] Calculated from the data of [16] in the presence of 10 mM *b*-glycerophosphate. [j] Determined at 5, 10, 20 and 40 mM glucose-6-P. [k] Determined under conditions similar to those in fig.2 with 0.05, 0.1, 0.2, 0.4 and 1 mM IMP. [l] Determined at 10, 20, 30 and 40 mM glucose. [m] Determined at 12.5, 25, 50 and 100 mM glucose. [n] Determined at 0.025, 0.05, 0.1 and 0.2 mM caffeine. [o] Determined at 0.5, 1, 2 and 4 mM caffeine. [p] Determined at 5, 10, 20 and 40 mM glucose-6-P. Glucose-6-P was found to be a non-competitive inhibitor for glucose-1-P and the inhibition constants were calculated from the replot of the intercepts vs inhibitor concentration

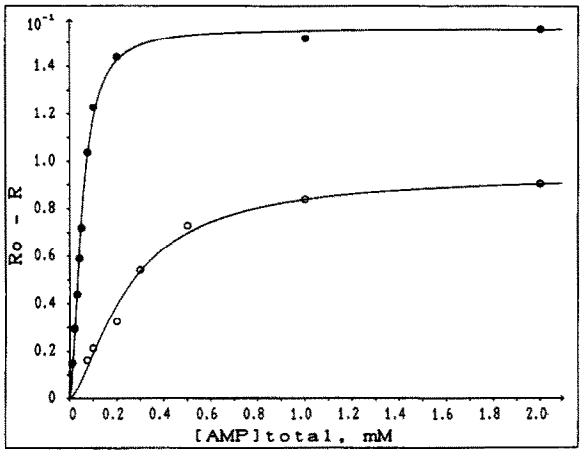

Fig.4. The effect of AMP on the ESR spectrum of spin-labelled phosphorylase *b* in the absence (○) or presence (●) of 0.9 M ammonium sulfate. ESR spectra were taken at pH 7.5 with 100 μM modified enzyme in 25 mM triethanolamine/HCl, 10 mM β-glycerophosphate, 50 mM KCl and 1 mM EDTA. R_0 and R represent the values of the ESR ratio at zero and at a given concentration of AMP.

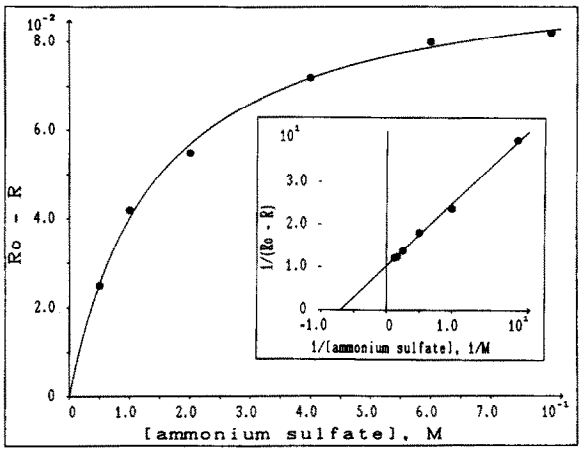

Fig.5. The effect of ammonium sulfate on the ESR spectrum of spin-labelled phosphorylase *b* (0.1 mM) in the presence of 0.1 mM AMP. The initial value of the ESR ratio was 0.64. Conditions are as in fig.4.

the presence of 0.8 M ammonium sulfate, the enzyme sedimented as a single sharp peak with an $s_{zo,w}) = 12.4$ S, a value similar to that of the tetramer [30]. The addition of 1 mM AMP did not change the aggregation state of the enzyme ($s_{zo,w} = 12.6$ S). Addition of caffeine (5 mM) to the ammonium sulfate-activated enzyme forced the enzyme to a species which is mainly dimeric ($s_{zo,w} = 9.8$ S). However, the tetramerization of phosphorylase *b* by ammonium sulfate is completely prevented in the presence of 50 mM glucose ($s_{zo,w} = 7.8$ S).

3.3. *ESR spectroscopy*

The ESR ratio of the spin-labelled phosphorylase *b* (0.66) was only slightly affected by concentrations of ammonium sulfate up to 0.9 M (0.67). Addition of AMP at concentrations up to 6.2 mM reduced the ratio to 0.56 as previously observed [20], but the ratio falls to 0.52 when AMP is added in the presence of ammonium sulfate. A plot of the change in the ESR ratio vs AMP concentration in the absence or presence of 0.9 M ammonium sulfate is shown in fig.4. The AMP-induced conformational change of the enzyme in the absence of ammonium sulfate is sigmoidal and following an approach similar to that of [20], we calculated an apparent dissociation constant K_d value of 200 ± 16 μM with a Hill coefficient of $n = 1.5 \pm 0.1$. However, in the presence of 0.9 M ammonium sulfate, an apparent dissociation constant K_d value of 4.0 ± 0.2 μM was calculated for the binding of AMP to the enzyme-ammonium sulfate complex and no homotropic cooperative effects were observed. To determine the effect of ammonium sulfate concentration on the affinity of the enzyme for AMP, the ESR spectra of the spin-labelled enzyme were recorded in the presence of various ammonium sulfate concentrations at a constant concentration of AMP (0.1 mM). The change in the ESR ratio plotted against salt concentration is shown in fig.5 from which a $K_{0.5} = 142 \pm 8$ mM for the salt effect can be calculated.

4. DISCUSSION

The results reported here show that ammonium sulfate modifies the catalytic, allosteric and structural properties of phosphorylase *b*. Under assay conditions, where the enzyme exists as a dimer [31], the presence of ammonium sulfate leads to: (i) an induction of AMP-independent activity; (ii) a stimulation of AMP- (or IMP-) dependent activity; (iii) a hyperbolic saturation curve for the binding of AMP, i.e. desensitization of allosteric interactions toward AMP; and (iv) reversal of the enzyme inhibition caused by glucose-6-P. Our results suggest that ammonium sulfate can be considered as an allosteric effector since it activates the enzyme through stabilization of its active conformation. A comparison of the catalytic properties of ammonium sulfate-activated phosphorylase *b* with those of phosphorylase *a* shows that the previous enzyme displays all the characteristics of phosphorylase *a*. It appears, therefore, that ammonium sulfate works as an activator in place of phosphate at the phosphorylation site at Ser-14. The self-activation of phosphorylase *b* by glucose-1-P [9], fig.1A) or phosphate [32], as well as the activation of the enzyme produced by high concentrations of several anions high on the Hofmeister series [9], could be attributed to the same phenomenon.

Crystallographic results on the R-state of tetrameric phosphorylase *b* demonstrate that sulfate binds at the serine phosphate site in a position close (2 Å) to the Ser-14 phosphate of phosphorylase *a* [14]. In this position, the sulfate is stabilized through interactions with

the hydroxyl group of Ser-14 and the guanidinium groups of Arg-69 and Arg-43 (from the symmetry-related subunit). As a result, a significant quaternary structural change is observed, which imposes tertiary structural changes on the subunits such that both a high-affinity AMP allosteric site and a phosphate-recognition catalytic site (by the movement of Arg-569) are developed [14]. In the presence of high concentrations of phosphorylase *b*, ammonium sulfate induces a change in the quaternary structure and by using a spin-label probe, which is very sensitive to conformational changes induced by AMP [20], it was possible to calculate an apparent dissociation constant value of 4 μM for nucleotide binding to the enzyme in the presence of 0.9 M ammonium sulfate. The tetrameric enzyme exhibits a high affinity for AMP in accordance with the predictions of the crystallographic result [14].

In conclusion, the kinetic results suggest that ammonium sulfate by itself does not quite trigger the true R-state conformation of the enzyme. Crystals of phosphorylase *b* in the presence of ammonium sulfate and AMP have been obtained (N.G. Oikonomakos et al., unpublished results) and will be analyzed to investigate in detail the site of interaction of phosphorylase *b* with AMP.

Acknowledgements: We are grateful to Dr L.N. Johnson and Dr D. Barford for critical reading of the manuscript and valuable suggestions.

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