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ON THE SUBUNIT STRUCTURE OF PARTICULATE AMINOPEPTIDASE FROM PIG KIDNEY *

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Summary

Solubilization of particulate aminopeptidase (EC 3.4.11.2) from pig kidney with Triton X-100 yields an aggregate (mol. wt. approx. 10^6) that decomposes into "free" aminopeptidase (mol. wt. 280 000) either upon autolysis at pH 5 or after exposure to trypsin. Both procedures yield free enzymes that are identical with respect to electrophoretic mobility, enzymatic activity and zinc content. After dissociation, the enzyme resulting from autolysis yields a single subunit of 140 000 molecular weight while the trypsin-treated enzyme produces three fragments (140 000, 95 000 and 48 000 mol. wt.). As the aggregate is formed by subunits 10 000 daltons heavier than those of the free enzyme, the existence of a hydrophobic portion anchoring the enzyme to the membrane might be postulated. Reactivation experiments carried out on the three purified fragments of urea-denatured aminopeptidase show that the 140 000 molecular weight subunit is the only one able to yield an active enzyme (after spontaneous dimerization). It can be concluded that the smaller fragments are artefacts resulting from trypsin degradation during purification.

Introduction

Particulate aminopeptidase from pig kidney (α-aminoacyl-peptide hydrolase, microsomal, EC 3.4.11.2) has a molecular weight of 280 000 and contains 2 g-atoms of tightly bound zinc per mol [2]. Both zinc atoms are undistinguishable with regard to involvement in catalytic activity and binding to the protein moi-

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ety [3,4]. On this basis, one could expect the enzyme to dissociate into an even number of subunits upon denaturation; instead, three fragments are observed. As particulate aminopeptidase had been routinely solubilized with trypsin [5], it was of importance to establish whether these fragments did not arise from trypsin attack during isolation.

Methods

Enzyme activity was analyzed spectrophotometrically using L-leucine-4-nitroanilide as substrate [2]. Protein was determined by the Lowry et al. [6] method or the Biuret [7] method, using bovine serum albumin as standard; for the purified enzyme, absorbance was measured at 280 nm ($E^{1\%} = 16.9$) [2]. Neutral sugars were assayed according to a modification [2] of the Winzler procedure [8]. Zinc analyses were performed on a Perkin-Elmer model 303 atomic absorption spectrophotometer.

Determination of unaggregated aminopeptidase. As aminopeptidase in the aggregated form (see below) does not penetrate 6% polyacrylamide gels, the fraction of the total activity of a sample that migrates through such a gel during isotachophoresis constitutes what will be called "free" aminopeptidase. A 6% polyacrylamide gel in 20 mM imidazole · HCl (pH 6.7) was polymerized to a height of 5 cm in an 8 mm (internal diameter) glass tube. An additional 15 mm segment filled with anode buffer (20 mM imidazole · HCl, pH 6.7) and closed by a dialysis membrane served as elution cell. The sample (3—4 enzyme units in 40% glycerol) was placed on top of the gel and the cathode buffer (0.5 M glycine/imidazole, pH 7.9) was layered on carefully. The electrophoreses were run at 2—3 mA per tube and the free enzyme was recovered from the elution cell after the run.

Reversible denaturation of aminopeptidase. The enzyme was denatured in 8 M urea, 6 M guanidine · HCl or 1% sodium dodecyl sulfate. For renaturation from urea and guanidine, the enzyme was added dropwise to a 15-fold excess of 50 mM Tris/acetate buffer, pH 7.2, 10 mM in EDTA [9] and dialyzed against the same buffer, concentrated by pervaporation and finally dialyzed against 20 mM Tris · HCl buffer, pH 7.2. Sodium dodecyl sulfate was removed by passage through a column of Dowex 1-X2 (formate form) in 0.05 M formic acid, 6 M in urea at pH 3.6 [10]. Renaturation was then achieved by dilution as described above.

Results

Preparation of aminopeptidase without trypsin treatment

The microsomal suspension [2] in 0.1 M Tris · HCl, pH 7.3, was solubilized by the addition of a 20% Triton X-100 solution; the final concentration was 0.5% protein and 2% Triton. The mixture was stirred gently at 4° C overnight. The activity does not sediment upon centrifugation (145 000 × g for 90 min). Solid $(NH_4)_2SO_4$ was added to 30% saturation, causing most of the Triton to separate in a floating layer which was discarded; aminopeptidase precipitated between 60 and 80% saturation. The pellet was dissolved in a minimal amount of 0.2 M sodium acetate buffer, 0.02% NaN_3, pH 5.0, and dialyzed against the

same buffer. The solution was incubated for 3 days at room temperature. Dissociation of the aminopeptidase aggregate was followed electrophoretically as described under Methods. Free aminopeptidase was precipitated between 70 and 75% $(NH_4)_2SO_4$ saturation. The pellet was redissolved and dialyzed against 0.02 M imidazole · HCl buffer, pH 7.2.

Final purification was achieved by means of isotachophoresis on a column (1.6 × 100 cm) of 4% acrylamide in a 0.02 M imidazole · HCl buffer, pH 6.7, which was also used as anode buffer. The cathode buffer was 0.5 M glycine/imidazole, pH 7.9. After a 24-h run at 4 mA, the portion of the gel containing aminopeptidase activity was removed and eluted electrophoretically.

The purified enzyme shows one band on polyacrylamide gel with a R_F identical to that of the enzyme solubilized with trypsin [2] (Fig. 1). In the presence of sodium dodecyl sulfate, it exhibits only one single band (corresponding to 140 000 mol. wt.) as compared to the three bands of the trypsin enzyme (Fig. 1). The R_F of the former band is identical to that of the slower migrating band of the enzyme solubilized with trypsin. Triton-solubilized amino-

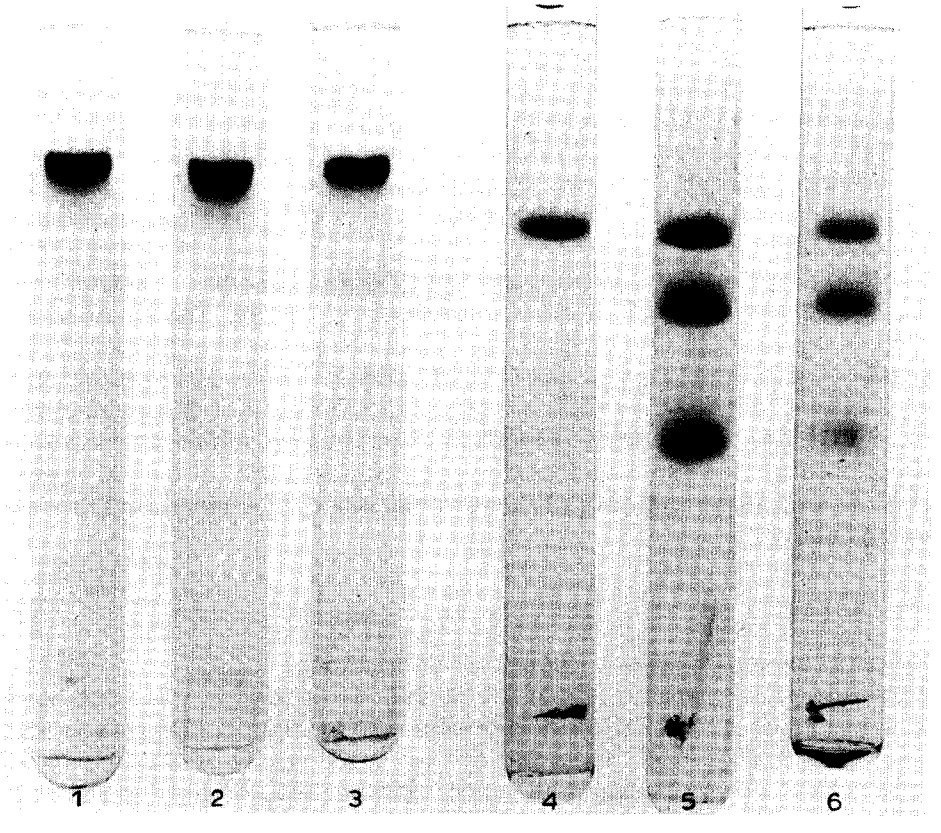

Fig. 1. Gel electrophoresis in the absence (tubes 1—3) and presence of sodium dodecyl sulfate (tubes 4—6). Pure Triton-solubilized aminopeptidase (mol. wt. 280 000) (tubes 1 and 4); aminopeptidase solubilized by the toluene-trypsin method (tubes 2 and 5); Triton-solubilized aminopeptidase treated by trypsin (tubes 3 and 6).

peptidase incubated with trypsin (even for a very short time) yields a preparation that displays the three band pattern characterisitic of trypsin aminopeptidase.

The elution volume from Sephadex G-200 is identical to that of the trypsin-solubilized enzyme (mol. wt. 280 000) [2]; so are the contents of zinc (2 g-atoms/mol), mannose and galactose. The enzymatic properties of both enzymes compare well also; the substrate specificities towards the 4-nitroanilides of leucine, alanine, glycine, phenylalanine, proline and half cystine, are identical. Urea, guanidine · HCl or sodium dodecyl sulfate completely inactivate aminopeptidase. However, the extent of reactivation that can be obtained upon renaturation is approximately twice as large for the Triton enzyme as for the trypsin-solubilized aminopeptidase (Table I).

Aggregated form of aminopeptidase.

When the 60—80% $(NH_4)_2SO_4$ pellet obtained after Triton solubilization is dissolved at pH 7.2 (20 mM Tris · HCl buffer) rather than at pH 5, aminopeptidase activity is eluted at 1.7 void volume of a Sepharose 4B column (Fig. 2A) as compared to 2.2. void volumes (Fig. 2B) after pH 5 incubation. The void volume of 1.7 corresponds to a molecular weight of approximately one million, instead of 280 000, suggesting that the enzyme is in an aggregated form. The preparation chromatographed at pH 7.2 is unable to dissociate at low pH. Similarly, if a heat treatment (60°C for 2 h) is performed before the pH 5 incubation, chromatography on Sepharose shows that the enzyme remains in the aggregated form (Fig. 2C).

The existence of two forms of aminopeptidase (aggregated and free) is also demonstrated by electrophoresis in 7.5% acrylamide gels (Figs. 2A and 2C): the aggregate is unable to enter the gel whereas the enzyme in the free form migrates as a single band with an R_F of 0.2. In the presence of sodium dodecyl sulfate, both forms of the enzyme yield a major band corresponding to a subunit of molecular weight close to 140 000. However, electrophoresis of a mixture of both forms shows a small migration difference corresponding to some 10 000 daltons (Fig. 3).

After a very short exposure to trypsin, the aggregate migrates during disc gel electrophoresis exactly as the free enzyme (Fig. 1). In the presence of sodium dodecyl sulfate, the three band pattern characteristic of trypsin-solubilized aminopeptidase [2] immediately appears.

TABLE I

PERCENT REACTIVATION AFTER DENATURATION OF AMINOPEPTIDASE SOLUBILIZED WITH TRITON OR TRYPSIN.

Renaturation was carried out as described in Methods. Activity of 30 units/mg corresponds to 100%.

Solubilization procedure	Denaturation agent		
	Urea (8 M)	Dodecyl sulfate (1%)	Guanidine · HCl (6 M)
Triton	79	56	25
Trypsin	36	27	14

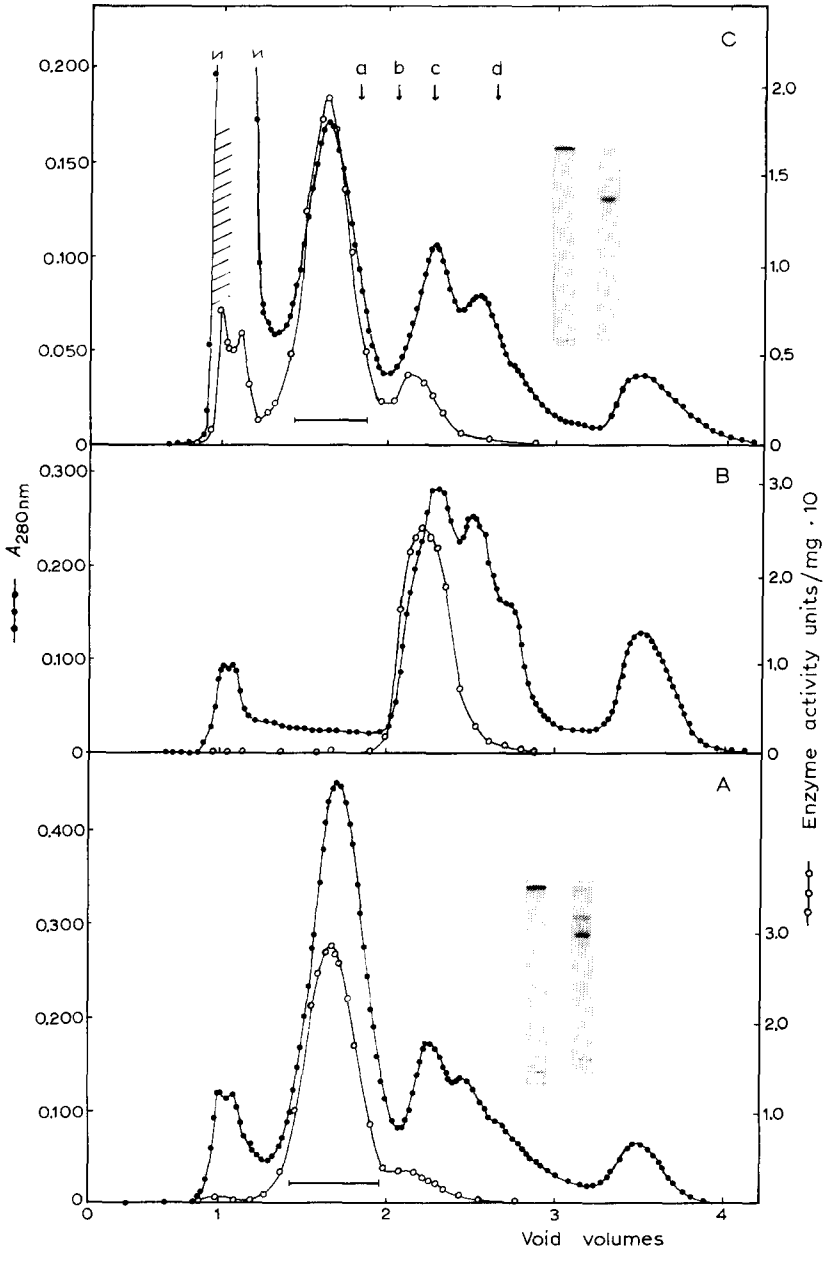

Fig. 2. Chromatography of Triton-solubilized aminopeptidase on Sepharose 4B. Elution profiles: A, sample maintained at pH 7.0 (180 units, 26 mg protein); B, incubated 3 days at pH 5 in sodium acetate buffer (130 units, 19 mg protein); C, after heat treatment (124 units, 14 mg protein). Disc electrophoresis of the activity peak (as indicated by horizontal bars) was performed in the absence and presence of sodium dodecyl sulfate. Column calibration by proteins of known molecular weights is indicated by arrows: a, thyroglobulin, mol. wt. 670 000 [15]; b, apoferritin, mol. wt. 467 000 [16]; c, aminopeptidase, mol. wt. 280 000 [2] and d, γ-globulin, mol. wt. 160 000 [17].

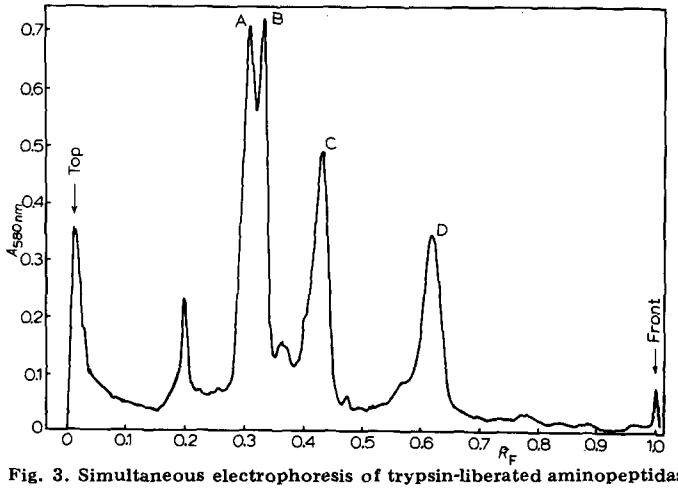

Fig. 3. Simultaneous electrophoresis of trypsin-liberated aminopeptidase and aminopeptidase aggregate in the presence of sodium dodecyl sulfate. A, subunit of aggregated aminopeptidase, B, C, D fragments of free trypsin-liberated enzyme.

Recombination of the subunits of Triton-solubilized aminopeptidase and of the fragments typical of the trypsin-solubilized enzyme

Gel filtration of trypsin-solubilized aminopeptidase, denatured in 8 M urea, resulted in the separation of three fragments (Fig. 4) of 140 000, 95 000 and 48 000 molecular weight, respectively; this is in good agreement with disc gel electrophoresis experiments [2]. Reactivation was performed on all three fragments separately, on a 1 : 1 : 1 mixture of the three fragments, and on urea-denatured Triton aminopeptidase. Taking the activity of the latter sample (10 units/mg) as 100%, the three fragments gave 90, 3 and 0%, respectively, whereas the mixture could only be reactivated to approx. 50%. After purification of the samples, all the activity was localized in a 280 000 molecular weight species (Fig. 5). The 95 000 and 48 000 molecular weight fragments apparently have no tendency to yield an enzymatically active protein.

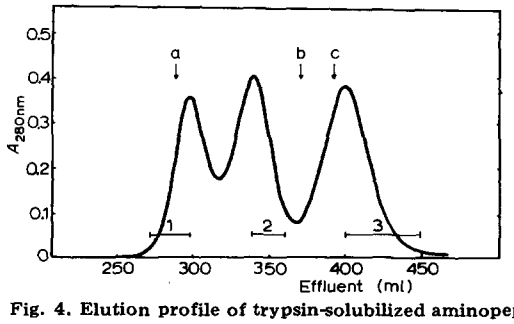

Fig. 4. Elution profile of trypsin-solubilized aminopeptidase on Sephadex G-200 in 8 M urea. The column (2.2 X 100 cm) was equilibrated and eluted with 50 mM formic acid (pH 3.6) 8 M in urea. Elution volumes of protein markers: a, γ-globulin, mol. wt. 160 000 [17]; b, bovine serum albumin, mol. wt. 68 000 [18] and c, ovalbumin, mol. wt. 43 000 [19] are indicated by vertical arrows. The fractions 1,2 and 3 collected as indicated by horizontal bars contained pure fragments of molecular weights of 140 000, 98 000 and 48 000, respectively.

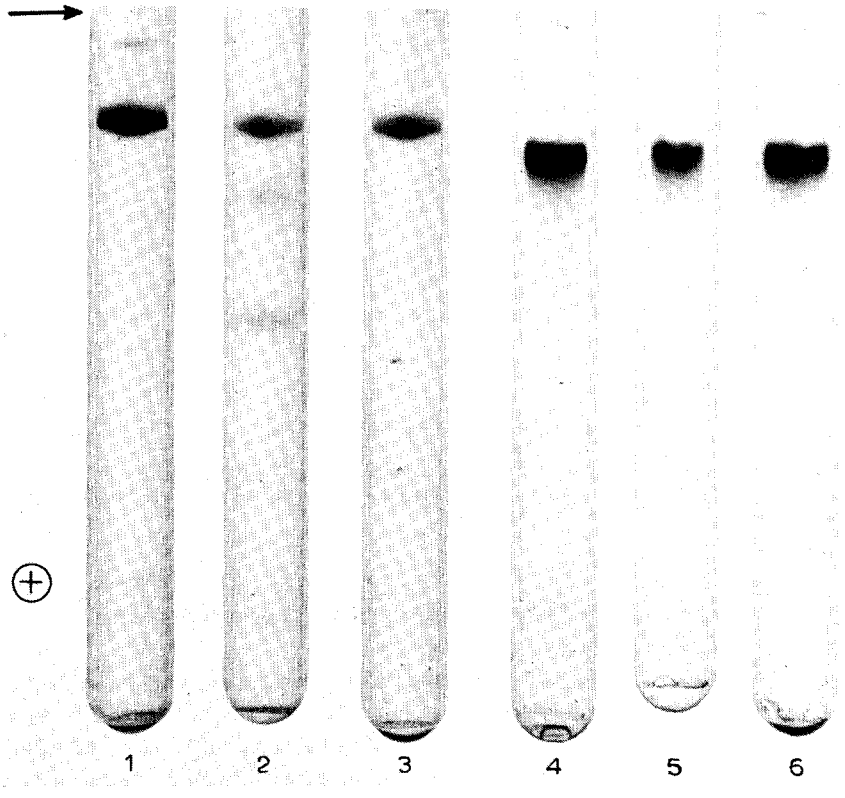

Fig. 5. Electrophoresis of aminopeptidase, after reconstitution from different fragments, in gels with (1—3) and without (4—6) sodium dodecyl sulfate. Free Triton-solubilized aminopeptidase (gels 1 and 4), active aminopeptidase reconstituted from a 1 : 1 : 1 (molar) mixture of fragments and purified (tubes 2 and 5), active aminopeptidase reconstituted from 140 000 molecular weight fragment only (tubes 3 and 6).

Discussion

Liberation of aminopeptidase from the membrane was achieved by means of Triton, as first proposed by Hanson [11]. This results in an aggregate with a molecular weight of approx. 10^6, which is able to accommodate 3—4 molecules of aminopeptidase (mol. wt. 280 000). Disaggregation can be achieved by the action of trypsin, or by incubation of the Triton extract at pH 5. This latter process seems to be an enzymatic one, as (1) autolysis of the aggregate shows a pH optimum, (2) a heat treatment prevents the decomposition of the aggregate; presumably the "pH 5 enzyme" is thermolabile, (3) chromatography on Sepharose 4B prevents disaggregation, as if the "pH 5 enzyme" had been separated from the aggregate.

Upon denaturation with sodium dodecyl sulfate, aminopeptidase, aggregated or free, dissociates into a single subunit (mol. wt. approx. 140 000). However, after autolysis (pH 5) or proteolysis (trypsin), the resulting subunit is somewhat smaller. It appears that a fragment corresponding to approx. 10 000 dalton has been lost. The latter might represent a hydrophobic anchor through

which the enzyme is attached to the membrane. This hydrophobic moiety might be responsible for the aggregation phenomenon; its loss yields an enzyme that does not aggregate any more. A similar situation has been described for cytochrome b_5 and cytochrome b_5 reductase, two other membrane-bound proteins [12,13].

In most respects, the properties of aminopeptidase are not affected by the choice of the solubilization procedure: the chemical composition is the same, i.e. metal and sugar content are identical, and so are specific activity and substrate specificity. Both preparations have the same molecular weight and cannot be distinguished by disc gel electrophoresis. Denaturation experiments, however, reveal a significant difference in the subunit structure: the preparation solubilized without trypsin dissociates into two subunits of identical molecular weight (140 000), whereas the trypsin-solubilized enzyme yields three fragments which certainly result from a peptide bond cleavage induced by the protease. The same pattern is observed after a trypsin attack on the Triton-solubilized enzyme, either in the aggregated or in the free form. This pattern appears after a very short exposure, suggesting that the molecule contains a bond that is very sensitive to trypsin. This is in line with reactivation experiments; the percentage of renaturation after dissociation by urea, sodium dodecyl sulfate or guanidine · HCl is consistently two times higher in the enzyme which has not been treated by trypsin, as if only the heaviest fragment could recombine into an active molecule. Further support for this view is provided by renaturation experiments performed on the different fragments isolated from trypsin-treated enzyme; only the heaviest fragment (140 000) dimerizes into an active aminopeptidase. As aminopeptidase possesses two independent zinc atoms of equal dissociation constant and catalytic importance [3,4], it appears likely that the enzyme is made of two identical polypeptide chains, each containing one active site.

Another pig aminopeptidase has recently been isolated from intestinal brush border [14]. On the basis of its molecular weight, sugar and metal content, it seems very similar, if not identical, to the kidney enzyme described here. However, it is highly probable that the three fragments observed on disc gels in the presence of sodium dodecyl sulfate do not represent the true subunits of the enzyme considering the sensitivity of this enzyme to trypsin. In the instance of the kidney, exposure of aminopeptidase to trypsin can be completely avoided; in contrast, endogenous trypsin is abundant in the intestine and contamination of intestinal brush border aminopeptidase by trypsin is likely.

Acknowledgements

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