κ -Casein Interactions in the Suspension of the Two Major Calcium-Sensitive Human β -Caseins

S. M. Sood,* G. Erickson,* and C. W. Slattery*,†

*Biochemistry Division, Department of Biochemistry and Microbiology

†Department of Pediatrics

School of Medicine, Loma Linda University

Loma Linda, CA 92350

ABSTRACT

The possible effects of both the β -casein (β -CN) phosphorylation level and the κ -CN glycosylation level on micelle formation were studied using the doubly-phosphorylated form (β -CN-2P) and the quadruply-phosphorylated form (β -CN-4P) of human β -CN, along with bovine κ -CN to compare with previous studies using the more highly glycosylated human κ -CN. Addition of bovine κ -CN to human β -CN-2P, β -CN-4P, or a 1/1 (wt/wt) mixture of the two was at κ/β molar ratios from 0.0 to ~0.6 and micelles were reconstituted by addition of Ca^{+2} either directly at 37°C for determination of the fraction suspended or at an initial temperature of 4° that was gradually increased to 37°C with the change in particle size monitored by turbidity measurements. Analysis of the data indicates that the 4P form requires more κ -CN for stabilization than the 2P form but that the mixture of the two is more like the 4P form in that lateral κ - κ interactions may enhance β - κ interactions and micelle formation. Above a κ/β molar ratio of about 0.2, the caseins were fully suspended into reconstituted micelles. However, micelle size decreased at a higher ratio, indicating that the κ -CN probably occupies a surface position and may regulate micelle size by its relative abundance. A comparison with published results suggests that the higher glycosylation level of human κ -CN may protect a larger surface area and result in smaller micelles. Changes in reconstituted micelle size with pH indicate that positively charged groups in the κ -CN may interact with the negatively charged phosphate esters in the β -CN moieties in addition to κ - β hydrophobic interactions.

(Key words: bovine κ -casein, human β -casein, reconstituted milk micelles, protein-ion interactions, protein-protein interactions)

Abbreviation key: β -CN-0P to β -CN-5P = phosphorylation level of human β -CN ranging from 0.0 to 5.0 as indicated by number preceding P.

INTRODUCTION

The suspended particles or micelles of milk are largely colloidal complexes of CN phosphoproteins. These complexes further bind and suspend necessary minerals, such as calcium phosphate, that would otherwise be precipitated and therefore not easily ingested by a newborn mammal. It is recognized that the CN-CN interactions in the formation of micelles probably result from a balance between electrostatic repulsion and hydrophobic interactions (Horne, 1998). The CN molecules containing a significant number of phosphate esters also carry a relatively large net negative charge and therefore interact with each other less readily until that charge is neutralized by binding Ca^{+2} ions or by a reduction in pH. Hydrophobic interactions take over and cause these “calcium-sensitive” casein molecules, in the absence of any stabilizing factor, to form large particles and precipitate. An increase in pH increases the net negative charge and prevents the precipitation. A decrease in temperature from that of the mammary gland, which reduces the hydrophobic interactions, has a similar effect. Micelles are formed when a stabilizing factor is present, usually in the form of a charged, glycosylated CN moiety that is not precipitated by Ca^{+2} and is at a concentration such that when it interacts with the complex to form a noninteracting surface, a colloidal suspension will be produced.

Although the CN systems of milk from different species all follow this same basic structural pattern, the components often differ in some of their details and may confer unique characteristics upon the milk, apparently customizing it for the requirements of the newborn infant. Of particular interest, there are a number of important differences in the CN from cow's milk and those from human milk. In the bovine system, the formation of CN micelles comes about through the stabilizing properties of κ -CN (Waugh and von Hippel, 1956) to

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Corresponding author: S. M. Sood; e-mail: ssood@som.llu.edu.

suspend the otherwise insoluble calcium-sensitive CN, mainly the α_{s1} -, α_{s2} - and β -CN fractions. Bovine κ -CN is glycosylated and contains about 5% carbohydrate by weight (Ruettimann and Ladiesch, 1987). It also contains two cysteines and normally exists in a multimeric structure formed by random disulfide linkages (Rasmussen et al., 1992). The existence of these disulfide-linked multimers appears to have little effect on the initial stabilizing ability of the κ -CN but may be important in maintaining the micelle system against changes in the environment (Talbot and Waugh, 1970). There is evidence to suggest that bovine κ -CN interacts with β -CN through electrostatic interactions involving the organic phosphates (Talbot and Waugh, 1970) but there are no doubt also some contributions from hydrophobic interactions (Hill and Wake, 1969).

In the human casein system, the calcium-sensitive CN fraction contains a very small amount of α_{s1} -CN (Rasmussen et al., 1995) but is mostly comprised of β -CN, a single protein phosphorylated to different degrees from zero (β -CN-0P) to five (β -CN-5P) (Groves and Gordon, 1970). The individual moieties may thus be used to examine the effect of protein phosphorylation on milk micelle formation. Much information may be obtained by comparing the doubly phosphorylated (β -CN-2P) and quadruply phosphorylated (β -CN-4P) forms, which are the major components of the β -CN fraction and together constitute nearly 70% of the total (Sood et al., 1985). Human κ -CN is glycosylated to about 55% of the molecule by weight (Dev et al., 1993) and contains only one cysteine residue. It could therefore theoretically form a disulfide-linked dimer but would not attain to the multimeric structure of bovine κ -CN. In particular, studies comparing its effects with those of bovine κ -CN should also give some indication of the influence of κ -CN glycosylation on micelle formation. We recently reported on the suspension, by human κ -CN, of each of the forms of human β -CN in the presence of 10 mM CaCl_2 (Sood and Slattery, 2002). There was an indication that human κ -CN also interacts by both electrostatic and hydrophobic bonding. Although stabilization was not eliminated for β -CN-0P, the suspension pattern was different from those entities with higher levels of phosphorylation (Sood and Slattery, 2002). To delineate the possible effects of κ -CN glycosylation, we now examine the stabilization and suspension of the 2P and 4P human β -CN moieties by bovine κ -CN both individually and in the 1/1 (wt/wt) mixture at two different total protein concentrations (3 mg/ml and 1.5 mg/ml) and in the presence of 5 and 10 mM Ca^{2+} (near physiological concentration).

Turbidity measurements on human β -CN-2P and β -CN-4P with added Ca^{2+} , both individually and mixed with bovine κ -CN, were also reported earlier (Sood et

al., 2002). The results suggested that the first step in micelle formation probably leads to polymers of limited size. In the present investigation we extend the turbidity measurements to include a 1/1 (wt/wt) mixture of the 2P and 4P forms of human β -CN with added bovine κ -CN. Because Talbot and Waugh (1970) suggested that the interaction of κ -CN could be dependent upon the charged phosphate esters and Parker and Dalglish (1981) observed an intricate relationship between the number of Ca^{+2} ions bound to β -CN and environmental conditions such as pH, measurements were also carried out at different pH values.

MATERIALS AND METHODS

Human milk was collected from donors at least 28 days postpartum and thus is mature milk rather than colostrum. The β -CN-2P and β -CN-4P were prepared using a slightly modified procedure of Groves and Gordon (1970) as previously described (Sood et al., 1985). Further purification was carried out by HPLC on Mono Q (Pharmacia LKB, Piscataway, NJ), which yielded virtually pure samples (Slattery et al., 1989). Bovine κ -CN was obtained from Sigma-Aldrich (St. Louis, MO).

A modification of the method of Malpress and Seid-Akhavan (1966) was used to study the suspension and stabilization of the different forms of human β -CN with bovine κ -CN in the presence of Ca^{+2} ions. The procedure, using human β - and κ -CN, was described by Sood and Slattery (2002). In the present case, each protein was dissolved in imidazole-KCl buffer (10 mM imidazole, 70 mM KCl) at the proper pH, to a concentration 2 $\times$ that of the final desired maximum concentration, as determined spectrophotometrically. A varied amount of bovine κ -CN solution (0 to 0.4 ml) was combined with a constant amount of β -CN (0.5 ml) to give final κ/β molar ratios ranging from 0.0 to about 0.6. The total volume was adjusted to 0.9 ml with buffer, as needed. Duplicate tubes were prepared. The mixtures were then incubated at 37°C for 15 min. Afterwards, 0.1 ml of CaCl_2 was added to each of the mixtures to give either a 5 or 10 mM final concentration of Ca^{+2} . These were then agitated in a 37°C water bath for 1 h. Next, the mixtures were centrifuged at 400 $\times g$ for 3 min at 37°C, a known volume of the supernatant (0.5 ml) was diluted with 2.5 ml of 0.05 M sodium citrate to bind the Ca^{+2} and solubilize any colloidal protein and the absorbance values (A_{280} and A_{320}) of this nonprecipitated or suspended CN were measured to give a value corrected for scattering, $A_C = A_{280} - 1.7 \times A_{320}$. The A_C values from duplicate tubes varied from each other by very little to as much as 10% in a few cases and the average values, $A_{C, Ave}$, were used. FS, the fraction of the β -CN that remained suspended in the supernatant, was then cal-

culated by Equation (1), the relationship indicated earlier by Sood and Slattery (2002).

$$FS = \frac{A_{C, Ave}(\beta, \kappa, Ca^{+2} \text{ supernatant}) - A_{C, Ave}(\text{total added } \kappa)}{A_{C, Ave}(\text{total added } \beta)} \quad [1]$$

The solubility of the β -CN with no κ -CN present may be represented by FS_0 . Consequently, the effect of the added κ -CN on the β -CN that would normally precipitate may be examined by defining the fraction (Y) of precipitated CN that is stabilized, by Equation 2.

$$Y = (FS - FS_0)/(1 - FS_0) \quad [2]$$

These data may then be analyzed with a Hill-type plot (Hill, 1910) according to Equation 3.

$$\log_{10} [Y/(1 - Y)] = h \log_{10} [\text{mol } \kappa / \text{mol } \beta] + \text{Constant} \quad [3]$$

The parameter h is the Hill Coefficient. The value of h is the slope of the plot of $\log_{10} [Y/(1 - Y)]$ versus $\log_{10} [\text{mol } \kappa / \text{mol } \beta]$ determined at $Y_{1/2}$, the point where $Y = 0.5$. The value of the κ/β molar ratio at $Y_{1/2}$, designated as $(\kappa/\beta)_{1/2}$, is a measure of the concentration of κ -CN that is needed to stabilize the β -CN against precipitation while the value of h is a measure of the cooperativity of the β - κ interaction. When $h = 1$, there is no cooperativity and the binding of κ -CN to β -CN is not affected by previous binding. When $h > 1$, binding of additional κ -CN molecules to a β -CN complex is enhanced by the initial binding.

In addition to measuring the fraction suspended, turbidity measurements of a 1/1 (wt/wt) mixture of human β -CN-2P and β -CN-4P with added bovine κ -CN at κ/β molar ratios of 0.2 and 0.4 were performed according to a standard procedure described previously for the individual components (Sood et al., 2002). In that procedure, all the proteins are incubated together at 4°C and Ca^{+2} is added to that mixture. Turbidity measurements are then made as the temperature is increased.

RESULTS

At the pH of human milk, pH 7, the fraction of β -CN-2P suspended by bovine κ -CN at two different protein concentrations and levels of Ca^{+2} are shown in Figure 1A as a function of the κ/β molar ratio. Similar results for β -CN-4P are shown in Figure 1B. The first thing to note is that as the κ/β ratio approaches zero for these proteins, the precipitation is almost complete at 10 mM Ca^{+2} and at the higher protein concentration but is

Figure 1. The fraction of (A) human β -CN-2P and (B) human β -CN-4P not precipitating (fraction suspended) at pH 7 and 37°C in the presence of $CaCl_2$ with various molar ratios of added bovine κ -CN. The plots are for 3 mg/ml β -CN at 5 mM Ca^{+2} ($\diamond$) or 10 mM Ca^{+2} ($\square$) and 1.5 mg/ml β -CN at 5 mM Ca^{+2} ($\triangle$).

somewhat dependent on the protein concentration at 5 mM Ca^{+2} . There is no precipitation until Ca^{+2} is added (Sood et al., 2002). The β -CN-2P is suspended more readily, with a $(\kappa/\beta)_{1/2}$ of about 0.02 and h equal to just over one for the protein in 5 mM Ca^{+2} . However, in 10 mM Ca^{+2} , the $(\kappa/\beta)_{1/2}$ is almost 0.06 and the h is 2.0 which is equivalent to the β -CN-4P at 5 mM Ca^{+2} . The 4P form at 10 mM Ca^{+2} has a $(\kappa/\beta)_{1/2}$ of about 0.09 with h equal to 2.3.

The effects of pH on the suspension of these entities by bovine κ -CN are shown in Figure 2A for β -CN-2P and in Figure 2B for β -CN-4P. As the pH is lowered from the normal value of pH 7 in each individual case, the β -CN-2P now has more of a tendency to form a precipitate, but is more readily stabilized by κ -CN ($(\kappa/\beta)_{1/2} = 0.015$, $h = 1.1$). Increasing the pH to 8.6 essentially solubilizes the 2P form without added κ -CN. The pH lowering to 6.4 seems to have little effect on the β -CN-4P protein ($(\kappa/\beta)_{1/2} = 0.06$, $h = 1.8$). An increase in pH to 8.6 apparently increases the repulsive charge on

Figure 2. The effect of pH on the suspension of 1.5 mg/ml of (A) human β -CN-2P and (B) β -CN-4P by bovine κ -CN in 5 mM CaCl_2 at 37°C. The plots are at pH 6.4 ($\diamond$), pH 7.0 ($\square$), and pH 8.6 ($\triangle$).

both molecules to the extent that there is very little precipitate, even without added κ -CN, at this CaCl_2 level. Addition of κ -CN does suggest, however, that there still may be some β - κ interactions but without any cooperativity ($(\kappa/\beta)_{1/2} = 0.04$, $h = 1.0$). The addition of 10 mM Ca^{+2} leads to the requirement for more κ -CN for stabilization and an increase in the cooperativity of the interactions ($(\kappa/\beta)_{1/2} = 0.09$, $h = 2.3$).

Figure 3 compares the ability of bovine κ -CN to suspend the individual proteins and a 1/1 (wt/wt) mixture of the two at different total protein concentrations and different concentrations of added Ca^{+2} . The mixture is similar to the individual entities in that there is a larger fraction of the protein precipitated at higher Ca^{+2} and/or protein concentrations. The mixture is intermediate in each case but behaves more like the 2P form at the lower concentrations of these variables (Figure 3A) and more like the 4P form at the higher concentrations (Figure 3B) in terms of the FS values. However, the interactions of the κ -CN to stabilize the precipitate were more like the lower-calcium 4P form, with $(\kappa/\beta)_{1/2} = 0.06$, $h = 1.5$ and $(\kappa/\beta)_{1/2} = 0.06$, $h = 2.5$ for the 5 and 10 mM Ca^{+2} , respectively.

Figure 3. A comparison of the stabilizing ability of bovine κ -CN for human ($\diamond$) β -CN-2P, ($\square$) β -CN-4P and ($\triangle$) a 1:1 weight ratio mixture of the two at 37°C and pH 7. (A) Total protein of 1.5 mg/ml with 5 mM Ca^{+2} added. (B) Total protein of 3 mg/ml with 10 mM Ca^{+2} added.

While the above measurements examine the final stage of reconstituted micelle development, turbidity measurements as a function of temperature look at casein aggregates at an early stage of formation. Figure 4A shows how the proteins in a 1/1 (wt/wt) mixture of β -CN-2P and β -CN-4P associate or aggregate at two different concentrations of Ca^{+2} and two different κ/β molar ratios. Comparison with similar measurements using the individual proteins (Sood et al., 2002) again suggests that the behavior of the mixture is intermediate between the two. However, the temperature-aggregation results at a κ/β ratio of 0.2 were closer to those of the β -CN-4P while the results at a κ/β ratio of 0.4 were closer to those of β -CN-2P.

The effect of pH on the aggregation of the proteins at the two different Ca^{+2} concentrations and a κ/β ratio of 0.4 is shown in Figure 4B. The largest differences are seen when the pH is reduced to 6.4. As discussed by Sood et al. (1998), the changes here and in Figure 2 are no doubt associated with changes in charge on

Figure 4. Turbidity (A_{400}) as a function of temperature for mixtures containing 1.5 mg/ml each of human β -CN-2P and β -CN-4P with bovine κ -CN. (A) κ -CN added at a κ/β molar ratio of either 0.2 (dashed lines) or 0.4 (solid lines). The measurements were made in either 5 ($\square$) or 10 ($\triangle$) mM Ca^{+2} . (B) κ -CN added at a κ/β molar ratio of 0.4 but at different pH values. The plots represent pH 6.4 (open symbols), pH 7.0 (shaded symbols) and pH 8.6 (filled symbols) at either 5 mM (squares) or 10 mM (triangles) Ca^{+2} .

the organic phosphate groups of the β -CN molecules since other charged groups are affected very little by pH changes in this range.

DISCUSSION

Using various physical measurements, we have now studied the self-association of the major individual Ca-sensitive moieties of human casein, β -CN-2P (Sood et al., 1992) and β -CN-4P (Sood and Slattery, 1997), as well as the mixture of the two (Sood and Slattery, 2001) in the presence and absence of Ca^{+2} . These were followed by a determination of the suspension of all of the individual moieties by human κ -CN (Sood and Slattery, 2002) and of the early steps of micelle formation when bovine κ -CN was added to each of the major human β -CN entities and the temperature was increased (Sood

et al., 2002). The results reported here now look at both the early steps of micelle formation for a 1/1 (wt/wt) mixture of the 2P and 4P forms with added bovine κ -CN, to look at the effects of glycosylation, as well as the amount suspended at 37°C at various κ/β molar ratios.

Figs. 1 and 3 indicate that above a κ/β molar ratio of 0.2, the caseins are fully suspended into reconstituted micelles. However, Figure 4A shows that at 37°C the reconstituted micelle size is quite different for ratios of 0.2 and 0.4, indicating that the κ -CN mostly occupies a surface position and may regulate the micelle size by its relative abundance. A comparison of these results with those using human κ -CN may be made. Stabilization of human β -CN entities by human κ -CN (Sood and Slattery, 2002) showed the same progression with protein phosphorylation as shown here with bovine κ -CN, suggesting a greater ability of the higher phosphorylation levels to cross-link by calcium bridges, as explained by Sood and Slattery (2002). However, the human κ -CN apparently stabilizes better than the bovine, attaining almost complete suspension of β -CN-2P at a κ/β ratio of less than 0.05 and of β -CN-4P at a ratio of about 0.1 (Sood and Slattery, 2002), agreeing with observations by Yamauchi et al. (1981) on the whole human β -CN fraction.

It is of interest that the cooperativity of the protein-protein interactions as measured by the Hill Coefficient increases with higher concentrations of Ca^{+2} , and these are in fact the conditions under which the protein aggregates are larger (Figure 4). Rather than indicating that there are β - β interactions that affect β - κ interactions, this probably reflects the additional κ - κ interactions that result in the κ -CN molecules being in a position to cross-link through S-S disulfide bonds and form dimeric or multimeric structures.

This difference between the stabilizing ability of bovine and human κ -CN is probably due to the large difference in glycosylation of the molecules. The κ -CN that resides on the surface of the milk micelles probably stabilizes them against aggregation by the extension of the negatively charged C-terminal portion of the molecule into the solvent to form a hairy layer (Walstra, 1990) or salted brush (de Kruif, 1999), even in the absence of glycosylation, because of the 8 negatively charged amino acid residues in the 45 at the C-terminus (Alexander et al., 1988). For comparison, human κ -CN contains only 6 negative charges in the 45 C-terminal amino acid residues (Edlund et al., 1996). In bovine milk, approximately half of the κ -CN molecules are glycosylated and there is apparently little difference in the portion glycosylated in different sized micelles (Dalgleish, 1985). One could assume that it is the average glycosylation that is important. The glycosylation occurs at threonine 131 (out of 169 amino acids) for

normal κ -CN and also at another (Thr 142) for colostrum κ -CN (Fiat et al., 1981). These oligosaccharides are capped by N-acetylneuraminic acid with a negative charge and may also contain this sugar residue in a branch of the oligosaccharide (Fournet et al., 1979). Human κ -CN contains up to 9 oligosaccharides (van Halbeek et al., 1985) and, like bovine κ -CN, may be more highly glycosylated in colostrum milk. Approximately 2.5% of the dry weight of bovine κ -CN may be released as neuraminic acid (Talbot and Waugh, 1970) whereas this sugar comprises about 7% of the human κ -CN (Dev et al., 1993). Thus, the area of the micelle surface that is influenced by the charged C-terminal macropeptide of a single κ -CN molecule may be larger when it is glycosylated and also has additional negative charges. This should lead to the same amount of κ -CN stabilizing many smaller casein micelles with larger total surface area, similar to the effect of the increase in κ -CN concentration seen here. This is also consistent with the fact that micelles in human colostrum have about a 50% higher κ/β (wt/wt) ratio (Cuilliere et al., 1999) and are even smaller in size than those in mature milk (Ruegg and Blanc, 1982).

As indicated in Figure 4B, changing the pH of the mixture of all three of these caseins has different effects on the formation of aggregates, depending upon the Ca^{+2} concentration. At the protein concentration used, increasing the pH from 7 to 8.6 almost eliminated aggregation at 5 mM Ca^{+2} but had little effect at 10 mM Ca^{+2} . Lowering the pH to 6.4 caused a significant increase in particle size at 37°C, characteristic of an increase in the effectiveness of the hydrophobic interactions. However, this increased particle size implies that micelles with a smaller total surface area are now being suspended and stabilized by the same amount of κ -CN, such as is indicated for the individual β -CN proteins by the results in Figure 2. If we still assume that an equivalent fraction of micelle surface coverage is required for suspension, this suggests that the equilibrium between κ -CN in the surface and that in solution may be shifted toward solution by the decrease in pH.

Talbot and Waugh (1970) suggested that the evidence for electrostatic interactions between κ -CN and the calcium-sensitive molecules to form micelles could be the result of interactions between amino groups on the κ -CN and the organic phosphates of α - and β -CN. This may be true in the human system as well. There would thus be two effects of changing the charge on the organic phosphates by changing the pH. At pH 8.6, the increase in negative charge on the organic phosphate groups could prevent β -CN aggregation, but there could still be a significant interaction with κ -CN amino groups. This may be partially overcome by reducing the net charge with Ca^{+2} binding at a higher Ca^{+2} concentra-

tion. At pH 6.4, the organic phosphate groups are partially discharged and may reduce both the κ - β and β - β electrostatic interactions but allow for increased β - β hydrophobic interactions. This emphasizes the balance between hydrophobic interactions, possible stabilizing salt bridges, and/or repulsive electrostatic interactions, in casein aggregation and micelle formation, as invoked in most micelle models, including those constructed from sub-micelles such as that described by Slattery and Evard (1973) and Slattery (1977) as well as the recent so-called "dual-binding" model of Horne (1998).

CONCLUSIONS

When compared with data obtained with highly glycosylated human κ -CN (Sood and Slattery, 2002), the results obtained here indicate that at a particular κ/β molar ratio, increased κ -CN glycosylation leads to a smaller average micelle size. Conversely, increased glycosylation would give the same average micelle size with a lower κ/β molar ratio. Analysis of the stabilization patterns indicates that individually, the 4P form requires more κ -CN than the 2P form for stabilization but at higher Ca^{+2} concentrations the mixture acts more like the 4P form. Under these conditions, interactions of the κ -CN molecules with the β -CN complexes are enhanced by previous κ -CN binding, suggesting that lateral κ - κ interactions may be involved. The formation of milk micelles through the suspension and stabilization of the Ca-sensitive caseins by κ -CN, leads to a structure in which the κ -CN in the surface prevents further micelle-micelle interactions. While full stabilization of human β -CN-0P, β -CN-4P and/or a 1/1 (wt/wt) mixture of the two with bovine κ -CN is achieved above a κ/β molar ratio of ~ 0.2 , the average size of the reconstituted micelles at 37°C is smaller at a higher molar ratio, indicating that micelle size is probably partially determined by the relative abundance of κ -CN. Since stabilization occurs for the more highly glycosylated human κ -CN at a κ/β molar ratio of ~ 0.05 and above, the extra negative charges from the carbohydrate of a glycosylated κ -CN molecule apparently protect a larger micelle surface area from interaction and would lead to smaller average micelle size for a given amount of κ -CN. The relative size of the reconstituted micelles as the pH is reduced to 6.4 or increased to 8.6 from the standard value of 7.0 suggests that the κ -CN may interact with the charged phosphate esters in the β -CN moieties as well as through hydrophobic bonding. A change in phosphate average charge with a change in pH could thus alter the surface-solution equilibrium distribution for κ -CN. These results are applicable to the building of milk micelle models and an understand-

ing of their structure and function as a component of human food.

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