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GLYCOSYLATION OF κ-CASEIN

I. LOCALIZATION AND CHARACTERIZATION OF SIALYLTRANSFERASE IN BOVINE MAMMARY GLAND

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Summary

A sialyltransferase (CMP-*N*-acetylneuraminate:D-galactosyl-glycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1) which attaches *N*-acetylneuraminic acid to the terminal end of the carbohydrate chain of κ-casein was found to be concentrated in Golgi apparatus-enriched fractions of bovine mammary gland. Maximum sialyltransferase activity was obtained at pH 5.5 and 37°C in the presence of 1 mM dithiothreitol and Triton X-100. A K_m of 0.19 mg asialo-κ-casein/ml (0.01 mM) was obtained for the sialyltransferase. Native κ-casein also served as acceptor for *N*-acetylneuraminic acid transferase of Golgi apparatus-enriched fractions although at a slower rate than did asialo-κ-casein. The sialyltransferase has a divalent cation requirement for maximum activity which was best satisfied by the presence of 10 mM Mn^{2+}.

Introduction

The major caseins of bovine milk, α_{s1}-, β- and κ-caseins, exist primarily in the form of micelles which range in size from 500—2500 Å [1]. κ-Casein is a secretory glycoprotein synthesized by the mammary gland and is the only casein which is soluble in solutions containing a wide range of Ca^{2+} concentrations [2]. Moreover, κ-casein possesses the ability to protect other caseins against precipitation by Ca^{2+}. Because of this unique Ca^{2+}-stabilizing ability, κ-casein has been assigned a prominent role in determining casein micelle stability [2].

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Abbreviations: NAcNeu, *N*-acetylneuraminic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

N-Acetylgalactosamine (GalNAc), galactose (Gal) and *N*-acetylneuraminic acid (NAcNeu) are the only carbohydrates found in κ-casein [3]. These carbohydrates apparently exist as trisaccharide moieties with the basic structure: α-NAcNeu—(2 → 3)—β-Gal—(1 → 3)—GalNAc [4]. However, some evidence exists for the attachment of additional *N*-acetylneuraminic acid and galactose to this basic trisaccharide moiety [5,6]. Basic trisaccharide moieties are attached to the C-terminal portion of κ-casein through O-glycosidic linkages between residues of threonine and *N*-acetylgalactosamine [4,5]. Part of the observed heterogeneity of κ-casein can be attributed to the varying amounts of basic trisaccharide moieties which are attached. 25—50% of κ-casein is carbohydrate free [7], while the remainder contains 1—5 trisaccharide moieties [7,8]. Similar heterogeneity has been observed in a number of glycoproteins [9—14].

There is evidence that the degree of glycosylation of κ-casein may have functional significance. Sullivan et al. [15] first suggested an inverse relationship between size of casein micelles and content of κ-casein, an observation since confirmed by others [16—18]. Although Thompson and Pepper [19] showed that removal of terminal *N*-acetylneuraminic acid from κ-casein reduced its ability to stabilize α_{s1}-casein against precipitation by Ca^{2+}, several other workers [20—23] have found no differences in the Ca^{2+}-stabilizing ability of carbohydrate-free and glycosylated κ-caseins. However, no indication of the stability of the resultant micelles was given in these studies [24]. Creamer et al. [25] separated casein micelles into two fractions, based upon size, and showed that while larger micelles contain proportionately less κ-casein than do smaller micelles, the κ-casein in larger micelles is more extensively glycosylated. They suggested that carbohydrate-free κ-casein is confined to the micelle surface while glycosylated κ-casein is located primarily in the micelle interior. These results prompted Slattery [26] to examine a more extensive separation of casein micelles into seven different size classes plus supernatant. Supernatant contained the highest proportion of glycosylated κ-casein while the largest micelles contained κ-casein with the highest weight-average molecular weight, the greatest amount of associated hexose and the highest proportion glycosylated, excluding supernatant κ-casein. With only one exception, the proportion of glycosylated κ-casein in the remaining micelle size classes appeared to be inversely related to micelle size. Slattery proposed that, during milk synthesis, the glycosylation of κ-casein occurs only after complete micelle formation so that surface or near-surface κ-casein is glycosylated while interior κ-casein remains unchanged.

This paper presents results of a study conducted to localize κ-casein sialyltransferse (CMP-*N*-acetylneuraminate:D-galactosyl-glycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1) in bovine mammary gland and to characterize this transferase activity.

Materials and Methods

Mammary tissue was obtained from lactating Holstein cows at slaughter and was transported to the laboratory on ice. Fractions enriched in Golgi apparatus and rough endoplasmic reticulum were isolated as described [27]. For comparative purposes, total microsomes [28] and smooth microsomes [28] were

also isolated from mammary tissue homogenates. Total particulate fractions were prepared from filtered homogenates by centrifugation at 120 000 $\times g$ for 1 h at 1°C [29]. Fractions were resuspended in 0.32 M sucrose/14 mM 2-mercaptoethanol and held at −20°C until used for sialyltransferase assays. Protein was determined according to Lowry et al. [30] with bovine serum albumin as standard.

Skim milk was prepared by centrifugation (7000 $\times g$ for 15 min at 23°C) of whole milk from the complete milking of an individual cow homozygous for the A variant of κ-casein. Casein was isolated from skim milk by acid precipitation at pH 4.6 and was washed twice with distilled water. κ-Casein was isolated by gel exclusion chromatography on Sephadex G-150 [31]. Purity was monitored by examination of fractions by polyacrylamide gel electrophoresis in 4 M urea at pH 9.6 [32].

Asialo-κ-casein was prepared by incubation of κ-casein with neuraminidase from *Vibiro cholerae* (Schwartz/Mann, Orangeburg, New York) at 37°C for 2 h. The incubation mixture contained 10 mg/ml κ-casein, 0.1 M sodium acetate (pH 5.5) 5 mM diisopropylfluorophosphate, 6 mM $CaCl_2$ and 50 I.U./ml neuraminidase. Released *N*-acetylneuraminic acid was measured by the thiobarbituric acid assay of Warren [33]. Under these conditions, more than 95% of the *N*-acetylneuraminic acid attached to κ-casein was routinely removed. Neuraminidase treatment exposed 0.19 μmol potential acceptor sites for *N*-acetylneuraminic acid/mg κ-casein.

Prior to use in sialyltransferase assay mixtures, it was necessary to separate the neuraminidase from asialo-κ-casein. Initially, neuraminidase was removed by passage through CM-Sephadex at 4°C according to Schachter et al. [34] except that the pH of the acetate buffer was 5.5. However, asialo-κ-casein collected from the column still retained small traces of neuraminidase activity and caused the sialyltransferase assay to be non-linear with time. Alternatively, neuraminidase was removed by affinity chromatography [35] using bovine submaxillary mucin (Sigma) bound to Sepharose 4B. Asialo-κ-casein was not retained by the mucin-agarose and was recovered by elution at 4°C with 0.02 M sodium acetate buffer (pH 5.0)/0.1 M KCl. However, trace amounts of neuraminidase were still present in preparations of asialo-κ-casein. As a result of difficulties encountered in removing small traces of neuraminidase, preparations of asialo-κ-casein (5 mg/ml) in 0.01 M Tris-HCl (pH 7.5) were heated at 80°C for 10 min to inactivate neuraminidase following elution from either CM-Sephadex or mucin-agarose.

Incubation mixtures for assay of sialyltransferase [34] contained 0.2 mg asialo-κ-casein, 73 nmol cytidine 5′monophospho-*N*-acetylneuraminic acid containing 0.3 nmol [^{14}C]cytidine 5′-monophospho-*N*-acetylneuraminic acid (Amersham/Searle, Chicago, IL, spec. act. 259 Ci/mol), 20 μmol 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 5.5, 200 nmol dithiothreitol, 0.025 μl Triton X-100 and 50 μl enzyme, in a final volume of 0.2 ml. Incubations were at 37°C for 20 min and reactions were stopped by addition of 100 μl 2% sodium tetraborate. Portions of reaction mixtures were spotted on Whatman No. 3MM paper strips which were developed (descending) overnight with 1% sodium tetraborate. Paper strips were air dried and segments around the origin, which contained the protein-bound *N*-[^{14}C]acetylneuraminic acid, were cut

out. Radioactivity in paper strips was determined in toluene-based liquid scintillation fluid. Sialyltransferase activity was expressed as nmol *N*-acetylneuraminic acid incorporated/mg membrane protein. Non-enzymatic incorporation of *N*-acetylneuraminic acid by asialo-κ-casein was determined and subtracted from the values presented.

Results

Subcellular fractions of bovine mammary gland were examined for their ability to catalyze the transfer of *N*-acetylneuraminic acid from cytidine 5'-monophospho-*N*-acetylneuraminic acid to exogenous asialo-κ-casein. Golgi apparatus-enriched fractions possessed the highest specific activity, 17.3 nmol *N*-acetylneuraminic acid incorporated/mg membrane protein, of any of the fractions examined (Table I). This represented a 13.8-fold enrichment in sialyltransferase activity over that of total homogenates.

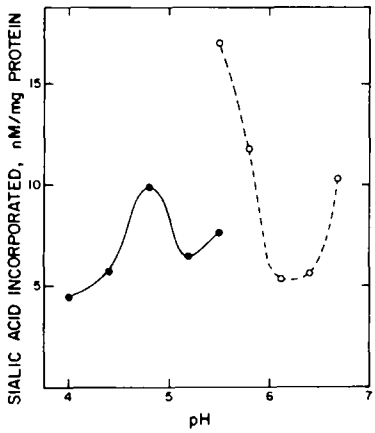
The effect of variation in pH on κ-casein sialyltransferase activity of Golgi apparatus fractions is shown in Fig. 1. Variations in pH between 5.5 and 6.7 were obtained with Mes, while sodium acetate was substituted to obtain pH values between 4.0 and 5.5. Maximum activity of sialyltransferase was obtained at pH 5.5 in Mes. Activities obtained at pH 4.0 and 4.3 are questionable since some component of the assay mixture (probably asialo-κ-casein) was insoluble. In addition, cytidine 5'-monophospho-*N*-acetylneuraminic acid is acid labile and undergoes hydrolysis below pH 6; at a pH of 4 hydrolysis is complete within 1 h at 37°C [36]. The effect of variation in temperature on sialyltransferase activity was also determined. Specific activities of 3.4, 6.2, 7.3 and 3.7 nmol *N*-acetylneuraminic acid incorporated/mg membrane protein were obtained after 20-min incubations at 23, 30, 37 and 45°C, respectively.

General characteristics of the sialyltransferase activity in Golgi apparatus fractions are given in Table II. The sulfhydryl reagent dithiothreitol was required for full activity. Omission of Triton X-100 from assay mixtures containing freshly isolated Golgi apparatus fractions resulted in an almost 50% decrease in activity. However, when Golgi apparatus fractions were frozen and thawed prior to assay, omission of Triton X-100 had little effect on sialyltransferase activity. Addition of 10 mM EDTA to the assay mixture resulted in a large reduction in transferase activity, indicating that the sialyltransferase

TABLE I

SUBCELLULAR DISTRIBUTION OF SIALYLTRANSFERASE IN FRACTIONS FROM LACTATING BOVINE MAMMARY GLAND

Subcellular fraction	Specific activity (nmol/mg protein)
Homogenate	1.2
Rough endoplasmic reticulum	2.0
Golgi apparatus	17.3
Smooth microsomes	5.4
Total microsomes	4.5
Total particulate	2.0



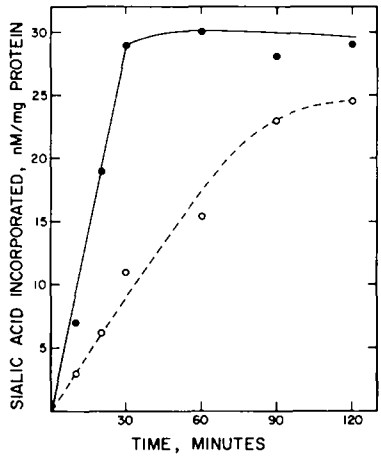


Fig. 1. Effect of variations in pH on κ-casein sialyltransferase activity in Golgi apparatus fractions from lactating bovine mammary gland. Sodium acetate (———) and MES (- - - - - -) were used to obtain variations in pH.

Fig. 2. Accumulation of NAcNeu by exogenous asialo- (———) and native (- - - - - -) κ-casein in Golgi apparatus fractions. Mixtures were incubated for the times indicated.

required endogenous divalent cations for maximum activity. When asialo-κ-casein was deleted, a small but measurable amount of endogenous transfer of *N*-acetylneuraminic acid was observed. Although the substrate(s) is unknown, the level of endogenous activity observed (4% of exogenous) is typical for this type of assay [34].

When sialyltransferase activity in Golgi apparatus-enriched fractions was assayed for varying time periods, reaction rates were linear for 30 min (Fig. 2). Initially, linearity could not be achieved due to small amounts of residual neuraminidase in preparations of asialo-κ-casein. However, linearity with time was observed when preparations of asialo-κ-casein were heated at 80°C for 10 min prior to use in sialyltransferase assays. Incorporation of *N*-acetylneuraminic acid was also obtained when native κ-casein was substituted for asialo-κ-casein in the sialyltransferase assay. Initial rate of incorporation with native κ-casein was somewhat slower; however, maximum accumulation (120 min incubation) of *N*-acetylneuraminic acid was 84% of that obtained with asialo-κ-casein.

TABLE II

REQUIREMENTS FOR SIALYLTRANSFERASE

Complete incubation mixtures contained the following components in final volumes of 0.2 ml; 0.2 mg asialo-κ-casein, 73 nmol CMP-NAcNeu containing 0.3 nmol [^{14}C]CMP-NAcNeu, 20 μmol MES, pH 5.5, 200 nmol dithiothreitol, 0.025 μl Triton X-100 and 50 μl of Golgi apparatus-enriched fraction. After 20 min at 37°C, the incubation mixtures were assayed.

Reaction mixture	Relative activity (%)
Complete	100
—Dithiothreitol	42
—Triton	53
+EDTA	22
—κ-Casein	4

When varying amounts of asialo-κ-casein were used in the assay mixture, sialyltransferase activity was observed to increase with increasing amounts of exogenous substrate (not shown). The concentration of asialo-κ-casein needed for half maximum activity was 0.19 mg/ml (0.01 mM) as calculated from double reciprocal plots.

Since addition of EDTA resulted in loss of sialyltransferase activity (Table II), the effect of variations in levels (2 and 10 mM) of several divalent cations on sialyltransferase activity was examined (Table III). Prior to sialyltransferase assay, Golgi apparatus-enriched fractions were dialyzed for 4 h at 4°C against 0.32 M sucrose containing 14 mM 2-mercaptoethanol with and without 1 mM EDTA to minimize the effects of endogenous cations. Dialysis had little effect on sialyltransferase activity in the absence of divalent cations since fractions dialyzed against EDTA incorporated 12.9 nmol *N*-acetylneuraminic acid/mg protein compared to frozen non-dialyzed fractions which incorporated 11.2 nmol *N*-acetylneuraminic acid/mg protein. In most cases (except 2 mM Zn^{2+} and Cu^{2+} and 10 mM Mn^{2+}) dialysis of Golgi apparatus fractions against EDTA resulted in lowered sialyltransferase activities (Table III). Although the concentration of EDTA was 1 mM, this effect was probably a combination of chelation of endogenous and exogenous divalent cations. Although low levels (2 mM) of all exogenous cations stimulated sialyltransferase activity, the greatest effect was obtained with Mn^{2+} in Golgi apparatus fractions both with and without EDTA. Similar results were obtained with higher levels (10 mM) of exogenous divalent cations. Again, highest activities were obtained with Mn^{2+} while addition of Zn^{2+} in the presence of EDTA exerted a slight inhibitory effect (Table III).

The effect of more extensive variations in cation concentration (2—30 mM) on κ-casein sialyltransferase activity was examined using Mn^{2+}, Ca^{2+} and Mg^{2+} (not shown). Addition of increasing amounts of Mn^{2+} resulted in corresponding increases in sialyltransferase activity to a maximum (118 nmol *N*-acetylneuraminic acid incorporated/mg protein) at 10 mM. Addition of Mn^{2+} in excess of 10 mM was accompanied by decreases in incorporation by asialo-κ-casein to a

TABLE III

DIVALENT CATION REQUIREMENTS FOR SIALYLTRANSFERASE

Complete incubation mixtures were as described previously with addition of 2 or 10 mM of each cation. Prior to assay Golgi apparatus-enriched fractions were dialyzed for 4 h at 4°C against 0.32 M sucrose containing 14 mM 2-mercaptoethanol in the presence (+EDTA) or absence (—EDTA) of 1 mM EDTA. Data represent the enzyme activity (nmol/mg protein).

Divalent cation	Cation (2 mM)		Cation (10 mM)	
	+EDTA	—EDTA	+EDTA	—EDTA
Co^{2+}	64.2	79.1	25.0	34.3
Zn^{2+}	17.8	16.5	7.9	16.6
Mn^{2+}	73.0	93.8	144.4	122.4
Mg^{2+}	43.9	75.8	56.2	56.4
Ca^{2+}	40.7	65.2	35.6	59.0
Sr^{2+}	43.4	85.5	57.1	78.6
Ba^{2+}	43.5	63.4	55.5	67.9
Cu^{2+}	52.3	36.1	18.9	46.2

level of 40 nmol *N*-acetylneuraminic acid/mg protein. Sialyltransferase activity in the absence of exogenous cations was 16 nmol *N*-acetylneuraminic acid incorporated/mg protein. Maximum sialyltransferase activities of 47 and 42 nmol *N*-acetylneuraminic acid incorporated/mg protein were obtained in the presence of 4 mM Ca^{2+} and Mg^{2+}, respectively. Addition of exogenous Ca^{2+} and Mg^{2+} (4 mM) produced an approximate 3-fold increase in sialyltransferase activity. Higher levels of both cations produced a gradual decrease to 22 nmol *N*-acetylneuraminic acid incorporated/mg protein at 30 mM.

Discussion

Golgi apparatus of lactating mammary gland appears to play a central role in synthesis and accumulation of milk constituents. Previously, Golgi apparatus-enriched fractions have been shown to contain a cyclic adenosine 3′,5′-monophosphate-independent protein kinase involved in phosphorylation of nascent casein polypeptides [37], a galactosyltransferase which participates with α-lactalbumin in synthesis of lactose [38,39] and an energy-dependent calcium accumulation system [40]. Results from this study indicate that the sialyltransferase which attaches terminal *N*-acetylneuraminic acid to κ-casein is also concentrated in Golgi apparatus-enriched fractions of bovine mammary gland. These results concur with the suggestion of Schachter et al. [34] that incorporation of individual moieties to the growing carbohydrate chain of secretory glycoproteins occurs sequentially as nascent protein chains move through the Golgi apparatus complex.

The carbohydrates which are attached to κ-casein occur primarily as trisaccharide moieties (see summary in ref. 6). However, Fournet et al. [6] have recently isolated a tetrasaccharide which contains an additional *N*-acetylneuraminic acid attached to the basic trisaccharide chain. The ability of native κ-casein to accumulate *N*-acetylneuraminic acid when incubated with Golgi apparatus-enriched fractions (Fig. 2) may represent binding of this additional *N*-acetylneuraminic acid to the basic trisaccharide moiety of native κ-casein.

Farrell [41] has proposed a series of steps which might occur during the bioassembly of casein micelles in the mammary gland. He has suggested that calcium accumulation occurs following phosphorylation of nascent casein polypeptides. κ-Casein, which plays an essential role in determining stability of casein micelles [2], not only contains phosphorus but is also the only casein which is a glycoprotein. Moreover, the degree of glycosylation of κ-casein appears to play a role in determining the size of casein micelles [16—18,25,26]. Slattery [26] has suggested that glycosylation occurs after formation of casein micelles. Studies to determine if phosphorylation necessarily proceeds the completion of glycosylation (attachment of *N*-acetylneuraminic acid) in Golgi apparatus and if glycosylation occurs after micelle formation are currently underway.

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