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Variation in the Glycosylation Pattern of Bovine κ-Casein with Micelle Size and Its Relationship to a Micelle Model†

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ABSTRACT: Bovine casein micelles were separated into seven size classes and the supernatant by differential centrifugation. The κ-casein was isolated from each class, and $s^0_{20,w}$, the weight-average molecular weight (M_w), the hexose content, and the proportion glycosylated were determined for each. Glycosylation appears to follow complete micelle formation so that surface or near-surface κ-casein is modified while that incorporated into the interior is unchanged. The largest micelles seem to have little interior κ-casein but instead act as a coat-core system with κ-casein all on the surface. The single class of largest micelles, fraction 1, contained κ-casein with the highest M_w, the largest amount of associated hexose and a proportion glycosylated approaching that of supernatant κ-casein. The proportion of glycosylated κ-casein in the remaining micelle size classes appeared to be inversely related to micelle size with the exception of fraction 2 which probably contained some fraction 1-type micelles. The hexose to glycosylated protein ratio generally followed the M_w and $s^0_{20,w}$ values. Further fractionation of the κ-caseins according to polymer size by gel filtration revealed that, except for the κ-casein from the largest micelles, hexose content and the hexose to glycosylated protein ratio were greatest for the larger sized polymers and decreased slightly as polymer size decreased. A higher hexose content for κ-casein polymers of high molecular weight than for those of lower molecular weight would favor a previously proposed model [Slattery, C. W., & Evard, R. (1973) *Biochim. Biophys. Acta 317,* 529].

The casein micelle system of bovine milk depends for its stability on the presence of κ-casein (Waugh & Von Hippel, 1956), but the stabilization mechanism has not yet been completely determined. Like the other caseins, κ-casein is a phosphoprotein, but dephosphorylation has little, if any, effect on its stabilizing ability (Pepper & Thompson, 1963). It is the only major casein that contains sulfhydryl groups and purified preparations contain disulfide-linked polymers ranging in size from trimers (Swaisgood & Brunner, 1963; Swaisgood et al., 1964) to decamers or larger (Talbot & Waugh, 1970). However, the extent of its association into covalent polymers has very little effect on the stabilizing capacity of the κ-casein (Talbot & Waugh, 1970). A constituent of κ-casein which does seem to have an effect on its stabilizing ability is the carbohydrate which may be associated with it. When κ-casein is glycosylated, the terminal sugar residue is very often sialic acid (Fiat et al., 1972). The sialic acid is, of itself, not necessary for micelle stability (Gibbons & Cheeseman, 1962) but desialylyzed κ-casein has less ability to stabilize α_s-casein against precipitation by Ca^{2+} ion than does intact κ-casein (Thompson & Pepper, 1962).

The relationship of carbohydrate to micelle structure was also explored by Creamer et al. (1973) who separated milk micelles into "large" and "small" by sedimentation and measured the extent of glycosylation of the κ-casein for these two size classes. They found a greater proportion of the κ-casein to be glycosylated in the large micelles than in the small and suggested therefore that the glyco-κ-casein probably was mainly in the micelle interior while the carbohydrate-free κ-casein was mainly on the micelle surface. This concept appears to be at variance with what is usually seen as a mechanism for stabilization by carbohydrate and calls for further investigation. This paper reports the results of more extensive

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experiments than those of Creamer et al. (1973). Bovine milk micelles were separated into several size classes by centrifugation and the glycosylation pattern for each class was examined. It appears that the largest micelles may have a different structure than the rest. The results favor a previously proposed model (Slattery & Evard, 1973).

Experimental Procedure

Fractionation of Skim Milk Micelles. Fresh pooled milk from the Loma Linda University herd was collected at the time of the afternoon milking and allowed to stand overnight at 4 °C to permit the cream to separate. Five-hundred-milliliter portions of the skim milk were then centrifuged at 13 700*g* according to the following protocol. The first centrifugation was for 30 min with the pellet designated as fraction 1. This pellet contained all of the micelles that could be completely removed by that centrifugation and decreasing amounts of all others in relationship to their sedimentation coefficients. The supernatant from that centrifugation was then centrifuged for 30 min longer than the previous time or 60 min and the pellet again collected. This process was repeated, with a 30-min increase in the time of centrifugation for each fraction, until seven pellet fractions were obtained. The final supernatant was then designated as fraction 8. Differential centrifugation in this manner does not yield size fractions containing only a certain range of micelle sizes but does give a decreasing average micelle size for each fraction as described by Waugh & Talbot (1971). The information obtained for each fraction thus reflects this average composition and should not be considered as applying to all micelles in the fraction. Experimental differences between these size fractions would consequently be less than if a size range were isolated. Electrophoretically pure κ-casein was isolated from each fraction by gel chromatography as previously described (Slattery & Evard, 1973).

Gel Chromatography of the κ-Casein from Each Fraction. Some of the κ-casein from each micelle fraction was itself fractionated according to covalent polymer size by rechromatography on Sephadex G-150 as described by Yaguchi et al. (1968). Five hundred milligrams of κ-casein from each fraction was applied to the column with the exception of that from fraction 4, for which there was only about 250 mg available. As the protein was eluted from the column in the TCU[1] buffer (0.005 M Tris, 6 M urea, titrated to pH 8.6 with citric acid), it was collected in 5-mL fractions. The κ-casein concentration in each tube was then determined by absorbance at 280 nm (Talbot & Waugh, 1970) and corrected for light scattering. Determinations of protein properties were made directly on the contents of each tube without any change in the buffer system.

Sedimentation Analysis. A Beckman Model E analytical ultracentrifuge equipped with schlieren and interference optics was employed in ultracentrifugal analysis. The temperature was controlled to within ±0.2 °C of 20 °C. Sedimentation coefficients were determined by least-squares analysis following the maximum ordinate of the schlieren patterns. The values for the sedimentation coefficient were corrected for protein concentration and for buffer density and viscosity. They will be reported in Svedberg units (S). Weight average molecular weights (M_w) were calculated by the method of Chernyak & Magretova (1975) from interference patterns obtained at sedimentation equilibrium. Separate determinations on the same sample were used to calculate a standard deviation for M_w.

[1] Abbreviations used: TCU, Tris-citrate-urea; Tris, tris(hydroxymethyl)aminomethane.

Determination of Total Hexose. The procedure of Dubois et al. (1956) was used to measure total hexose in the κ-casein samples. The hexose of κ-casein is mostly galactose (Fiat et al., 1972) but there may also be some mannose (Wheelock & Sinkinson, 1973), probably in a ratio of about 1:4 or 1:5 mannose:galactose in pooled milk. It can vary considerably for individual cows. Because of the higher absorbancy of mannose (Dubois et al., 1956), samples with the same amount of total hexose could appear to be different if there were a difference in the ratio of these two hexoses. However, plots of the absorbance due to hexose vs. concentration at different dilutions for each sample revealed essentially no difference in slope from one sample to the next. This is indicative of a constant ratio of mannose:galactose throughout the system. Amino sugars do not interfere with this determination and measurements at varying protein concentrations for the same sample revealed no effect due to protein.

Total hexose could be measured directly on the samples in the TCU buffer as they emerged from the G-150 column with no problems due to the high concentration of urea, but evidently an interfering carbohydrate was released from the G-150 by the TCU buffer and accumulated when buffer was not flowing. This made it necessary to wash the column thoroughly with buffer before applying the sample. Variation in the phenol concentration was corrected for by measuring a standard galactose solution along with the samples each time. The results will be reported as the ratio of the absorbance at $\lambda = 490$ nm (A_{490}) from the hexose determination to the corrected absorbance due to protein (A_{280}) in the same sample. This ratio will be referred to as the "hexose to protein absorbance ratio" in the remainder of the paper.

Electrophoretic Analysis. Samples of κ-casein in TCU buffer were mixed with 2-mercaptoethanol in a volume ratio of 2 to 1 and allowed to stand for 4 h before application to the gel for electrophoresis. A 7% polyacrylamide gel was used in the Beckman Microzone apparatus. The buffer used was 0.037 M Tris, 0.29 M glycine, 4.5 M urea and pH 8.4. A constant 310 V was applied to the polyacrylamide gel slab for 2.5 to 3 h with the current varying between 44 and 50 mA. The gel was then cut into strips for scanning at 280 nm on a Gilford spectrophotometer with an integrating recorder. The percentage of κ-casein appearing in different electrophoretic bands was then determined, and the proportion glycosylated, that which appears in all but the two slowest bands, was calculated.

Results

Properties of the κ-Casein from Each Micelle Fraction. The micelle size fractions studied here were obtained by differential centrifugation of cold milk. As shown by Rose (1968) and Downey & Murphy (1970), considerable β-casein and some κ-casein dissociates from the micelles into the serum in the cold. Apparently, however, the micelle sizes stay the same but the sedimentation coefficients decrease due to the loss of material (Lin et al., 1972). It would be reasonable to assume that, since the major material dissociating is β-casein, the same percentage is lost from all micelles and the density of all micelles thus remains equivalent. Under these conditions, differential centrifugation still results in a proper size fractionation but the κ-casein composition of the micelle fractions could be slightly different.

The data of Rose (1968) can be used to estimate the effect of κ-casein leaving the micelles upon the measurements reported here. Approximately 10% of the κ-casein is in the serum of warm milk and this increases to 20% in the cold. Thus, while the κ-casein content of the supernatant fraction would double, that of the micelles would decrease by only 1/9 or 11%. The

TABLE I: Characteristics of κ-Casein from the Various Micelle Fractions.

Fraction	$s^0_{20,w}$ (S)[d]	$M_w \pm$ SD[a]	Hexose:protein absorbance ratio	Proportion glycosylated	Hexose:glycosylated protein absorbance ratio[b]
1	3.82	319 000 ± 3000 (3[c])	0.096 ± 0.004[a]	0.50	0.192
2	3.52	311 000 ± 8000 (4)	0.067 ± 0.002	0.44	0.152
3	4.12	220 000 ± 3000 (4)	0.066 ± 0.001	0.36	0.183
4	4.54	286 000 ± 4000 (5)	0.078 ± 0.005	0.38	0.205
5	4.77	200 000 ± 3000 (5)	0.074 ± 0.003	0.40	0.185
6	4.68	274 000 ± 3000 (4)	0.067 ± 0.005	0.39	0.172
7	4.20	260 000 ± 9000 (4)	0.077 ± 0.004	0.47	0.164
8	4.24	251 000 ± 3000 (4)	0.078 ± 0.001	0.52	0.150

[a] Standard deviation. [b] Obtained by dividing column 4 by column 5. [c] The figures in parentheses indicate the number of determinations used to get the standard deviation. [d] Both the sedimentation coefficient and weight-average molecular weight for each fraction were determined in TCU buffer (see text).

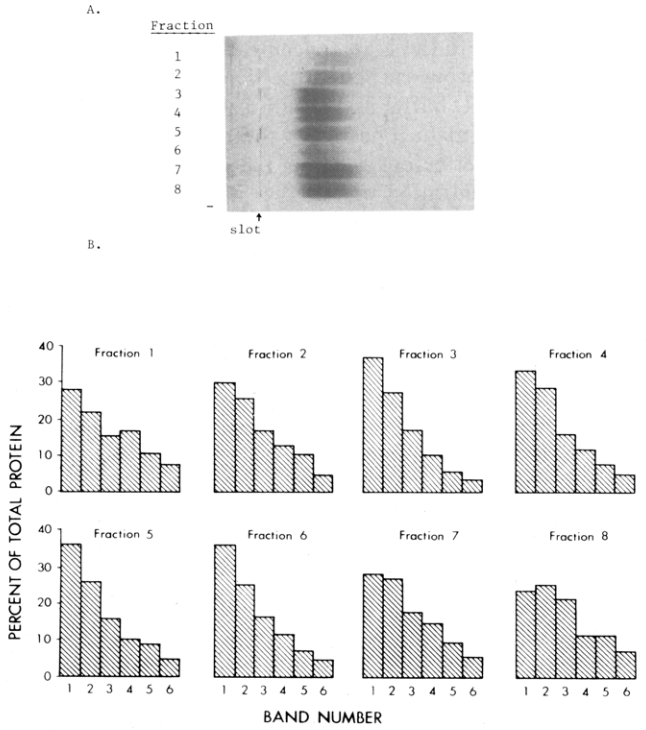

FIGURE 1: (A) Polyacrylamide gel electrophoresis patterns for the reduced κ-casein from each of the micelle size fractions. (B) Percent of total protein in the electrophoretic bands of each micelle size fraction. Band number 1 has the lowest mobility and band number 6 has the highest mobility in each case.

properties of the cold-dissociated κ-casein are probably not extremely different than the average found for the supernatant fraction and this can then be translated into a possible 3% to 5% difference in the values measured and reported below. This is close to the error in the measurement and would have little effect on the conclusions drawn.

A summary of the results of sedimentation analyses, hexose measurements, and the proportion glycosylated is given in Table I. A few general observations may be made. Fraction 1 and 2 κ-caseins appear different from the rest in that they have relatively low $s^0_{20,w}$ values but high average molecular weights. Both $s^0_{20,w}$ and M_w then increase and decrease together except for the anomalous M_w for fraction 5 κ-casein. The reason for that low value is not obvious since its elution curve from Sephadex G-150 indicates that it should be higher than the fractions on either side of it, in keeping with the $s^0_{20,w}$. The hexose to protein absorbance ratio is significantly higher for fraction 1 κ-casein but seems to follow no real pattern for the rest.

Polyacrylamide gel electrophoresis of the κ-casein from each micelle size fraction resulted in six major bands. A gel pattern stained with Amido Black 10B and the percentages of protein present in each band are shown in Figure 1 for the κ-casein from each fraction. Mobility of the bands increases from left to right. The two slowest bands probably represent genetic variants of κ-casein with no sialic acid (Pujolle et al., 1966; Schmidt et al., 1966), while the faster bands are due to increasing molecular charge when sialic acid is present. Examination of Figure 1 shows that the percent of total protein in the two slowest bands first increases and then decreases in roughly the same fashion as the $s^0_{20,w}$ values. Since the bands that contain sialic acid presumably also contain all the carbohydrate (Woychik et al., 1966), it appears that the carbohydrate may reduce the sedimentation velocity. The relative amount of protein in the four fast bands is the proportion glycosylated as given in column 5 of Table I. Dividing this value into the hexose to protein absorbance ratio of column 4 gives a hexose to protein absorbance ratio for just the glycosylated protein. This value is shown in column 6.

Properties of Rechromatographed κ-Casein. The κ-casein obtained from each micelle fraction, after being characterized as indicated in Table I and Figure 1, was rechromatographed on Sephadex G-150 and eluted with TCU buffer, resulting in the separation of the κ-casein from each size fraction into a near-continuum of polymer sizes. Such a size separation was indicated by a continuous decrease in sedimentation coefficient in each rechromatographed fraction as the elution volume increased. The elution curves themselves are revealing. The curve for the κ-casein from fraction 1 showed some asymmetry with a very slight hump or shoulder on the trailing side of the peak. This shoulder became more and more pronounced for the κ-casein from fractions 2 through 4 as shown by the elution curve for fraction 3 κ-casein in Figure 2. However, the elution curve for the fraction 5 κ-casein, also included in Figure 2, was almost symmetrical and fraction 6 through 8 κ-caseins showed only slightly more asymmetry. This probably means that the κ-casein from the larger micelles has a wider range of molecular weights than that from the smaller micelles. The asymmetry may be partly due to a difference in elution volume between glycosylated and nonglycosylated κ-casein (Andrews, 1965), but the hexose to protein absorbance ratios for the eluted materials indicate that this is probably not a major factor.

Figure 3 shows the hexose to protein absorbance ratios as

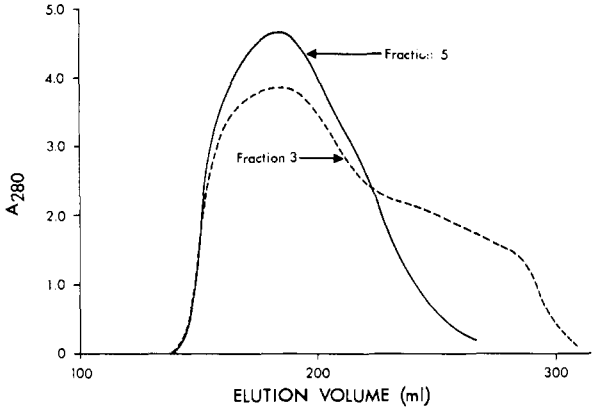

FIGURE 2: Protein absorbance vs. elution volume for fraction 3 (dashed line) and fraction 5 (solid line) κ-casein. The κ-casein was eluted from the Sephadex G-150 column (100 × 2.5 cm) with TCU buffer, pH 8.6 (see text), at a flow rate of 10 mL/h.

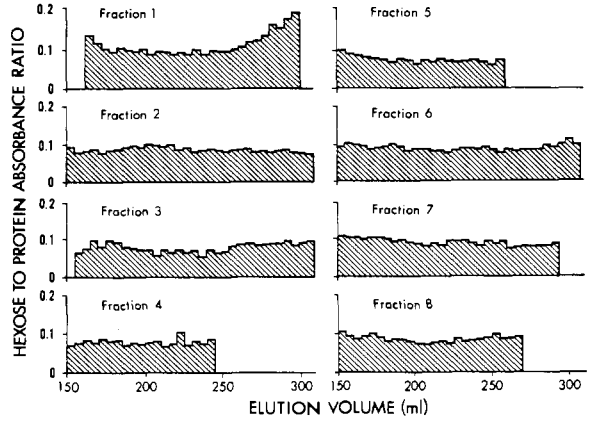

FIGURE 3: Hexose to protein absorbance ratio vs. elution volume for the κ-casein from each micelle size fraction.

a function of elution volume for the κ-casein from each fraction. Trends are small but noticeable in some cases and are reproducible from sample to sample. The κ-casein from fraction 1 exhibited the most striking variation with both high and low molecular weight material containing more hexose than the average. The hexose decreased in the low molecular weight κ-casein of fraction 2 (high elution volume), while that portion of the κ-casein of fraction 3 had more hexose associated with it. The hexose of fraction 4 through 8 κ-caseins either did not change significantly or decreased slightly with the elution volume.

The contents of several of the tubes collected upon the elution of the κ-casein from each micelle size fraction were subjected to polyacrylamide gel electrophoresis. Quantitative determination of the protein in each band revealed that the amount of protein that contained sialic acid and thus migrated the fastest generally followed the hexose to protein absorbance ratios. In general, sialic acid was associated with a larger percentage of the material eluting from the column first than with that eluting later. However, with the exception of the earliest tubes from fractions 3 and 4, the percentages did not decrease as fast as the amount of hexose did with the result that the hexose to glycosylated protein ratios decreased with increasing elution volume (or decreasing average molecular weight). These were only checked over the first portion of each elution curve and so later trends are unknown.

Discussion

The order of events as they occur during bovine casein micelle formation is still uncertain as is the structure of the micelles themselves. However, the data presented here are consistent with the following hypothesis. After protein synthesis, the ionic environment is such that α_s-, β-, and monomeric κ-caseins (Talbot & Waugh, 1970) aggregate first to form submicelles of variable composition and these aggregate further to give complete micelles (Slattery & Evard, 1973). It is possible that disulfide-linked covalent polymers of κ-casein could be formed before submicelle formation but it seems unlikely. If it is assumed that the postribosomal κ-casein must associate only with other κ-casein molecules for -SH oxidation, some sort of mechanical separation from the other caseins would be required until the polymers were formed. Otherwise, since κ-casein prefers to associate with α_s-casein rather than other κ-casein molecules (Waugh & Noble, 1965), submicelle formation would begin in preference to κ-casein aggregation. Also, the proposal of Talbot & Waugh (1970) for the participation of monomeric κ-casein in the initial aggregation is supported by calculations of micelle size distributions (Slattery, 1976) carried out under the assumption that κ-casein is monomeric. Oxidation of the -SH groups to form disulfide-linked covalent polymers could then occur either after submicelle formation or after the formation of the complete micelle, perhaps not until exposure to air after milking.

If, then, there are no covalent polymers prior to submicelle formation, there are three levels at which κ-casein glycosylation could take place: monomer, submicelle, or complete micelle. Glycosylation at the monomer level should result, if given enough time, in all of the molecules being fully glycosylated or at least in distributions the reverse of those shown in Figure 1. In order to obtain the distributions found, a short glycosylation time would have to be postulated. This could be possible if glycosylation stopped upon submicelle formation. One would further expect a random incorporation of glycosylated and nonglycosylated κ-casein into the submicelle (Slattery, 1976). This, however, would not result in a difference in the glycosylation pattern with micelle size as is shown in Table I. Consequently, it becomes necessary to first assume a nonrandom incorporation of glyco-κ-casein into the submicelle and then a nonrandom incorporation of these submicelles into the micelle itself, depending upon micelle size.

Similar problems arise if glycosylation occurs at the submicelle level. Again, a nonrandom glycosylation process would be needed with some submicelles being more highly glycosylated than others, followed by a size-dependent aggregation of the different types of submicelles. It seems rather that the data would be mostly easily accounted for by the construction of a micelle from submicelles so as to put some κ-casein in the micelle interior, such as with the model of Slattery & Evard (1973), followed by the addition of the carbohydrate. The results of Thompson & Pepper (1962) cited in the introductory section would tend to support a surface position for most of the carbohydrate. Furthermore, Fox & Morrissey (1972) indicate that disruption of the micelle by removing phosphate only increases the amount of sialic acid-containing κ-casein which is accessible to rennin attack from 60% to 70%. Even if rennin can penetrate into the micelle somewhat, a surface or near-surface position for sialic acid, and thus for essentially all the carbohydrate, seems likely (Woychik et al., 1966).

The data of Table I also support a surface position for the carbohydrate. The proportion glycosylated is highest for fraction 8 κ-casein, that in the supernatant. It decreases as the micelles get larger, suggesting that a smaller proportion may be available for glycosylation as the micelles get larger in accord with the model of Slattery & Evard (1973). There is, in

confirmation of the results of Creamer et al. (1973), some increase in the proportion glycosylated for the fraction 2 κ-casein and a larger increase for that from fraction 1, almost to the proportion for fraction 8. Recognizing that about half of the supernatant κ-casein may have come from the micelles and most probably from near the surface, I would propose that there may be a difference in structure between the largest micelles and the smaller ones. In this behavior, the fraction 1 micelles act as if they were a "coat–core" system, similar to that proposed by Waugh & Noble (1965), with a core of α_s- and β-caseins surrounded by the κ-casein, all in the surface. The increase in the proportion glycosylated in fraction 2 is probably due to the presence of some of these micelles in that fraction. Such a possibility would explain the characteristics of the reconstituted systems examined by Waugh & Talbot (1971) in that over half of the material in the systems they were studying was in micelles as large or larger than the largest micelles in skim milk. In their system there were strong indications that the micelles existed as a coat–core structure. These results seem to confirm those observations for such large micelles but suggest that there is a different structure for the majority of micelles present in natural systems.

Further support is given for the model of Slattery & Evard (1973) and for surface glycosylation by the data of column 6 of Table I. The hexose to glycosylated protein ratio, shown there, can be seen to generally follow the M_w and $s^0_{20,w}$ values. The main inconsistency in the pattern is in the low M_w for the fraction 5 κ-casein. In the model, κ-casein in the surface submicelles is more highly associated with other κ-casein and thus is more apt to form covalent disulfide-linked polymers of high molecular weight. These would be the ones to be glycosylated. Although all polymer sizes could be represented in the surface, as the degree of polymerization and the molecular weight decrease, an interior position for the κ-casein becomes more likely with less possibility for glycosylation. This is the only model which predicts that there would be more hexose associated with κ-casein in higher molecular weight polymers and then only if there is surface glycosylation subsequent to micelle formation.

Similar conclusions can be drawn from the measurements on hexose content and the proportion glycosylated as a function of the κ-casein polymer molecular weight in each fraction. As indicated earlier, the hexose to glycosylated protein ratios again seem to follow the polymer molecular weight as reflected by the elution volume. So far as is known, no other model can account for such changes.

Acknowledgments

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