A Protein Kinase is Located in the Micellar Fraction of Fresh Pasteurized Skimmed Farm Milk

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ABSTRACT

Recombinant protein kinase CK2 from the yeast *Schizosaccharomyces pombe* is able to phosphorylate casein in skimmed pasteurized milk. We could incorporate up to 540 pmol of phosphate into 50 μ g milk proteins, i.e., 0.26 P/mol caseins. To better understand the action of protein kinase CK2 on milk proteins, we have compared the action of *rspCK2 α* on milk, and on different casein micellar subfractions isolated from milk by ultracentrifugation. In contrast to the situation observed with phosphocaseinate, α_s casein was the best substrate for *rspCK2 α* , whether milk or micellar fractions were used as substrates. We have characterized the protein content of different micellar fractions obtained by ultracentrifugation of cow milk using capillary zone electrophoresis. We confirm that the κ casein content of micelles largely decreases when their size increases. In contrast, the α_s casein content slightly increased with micelles size and β casein content remained constant. All of the micellar fractions were substrates for *rspCK2 α* , but a significant amount of intrinsic protein kinase activity was also found. The intrinsic protein kinase used added ATP as phosphate donor, and was only slightly sensitive to high heparin concentration. It could phosphorylate micellar casein in milk ultrafiltrate, in the absence of addition of any metallic cofactor. Its activity was only slightly affected by the addition of either $MgCl_2$ or $MnCl_2$. $CaCl_2$ activated the enzyme significantly. The intrinsic kinase lost its activity with time, and could incorporate from 9 to 26% of the total phosphate incorporated in the presence of *rspCK2 α* . α_s casein was the best substrate of the intrinsic kinase, followed by β casein. In the presence of $CaCl_2$, the intrinsic kinase was found to incorporate up to 470 pmol of phosphate into 50 μ g of milk proteins.

(**Key words:** Milk, caseins, protein kinase, phosphorylation)

Abbreviation key: CZE = capillary zone electrophoresis, *rspCK2 α* = α subunit of recombinant *Schizosaccharomyces pombe* protein kinase CK2.

INTRODUCTION

Caseins have been studied for more than a century (Hammarsten 1883). Their primary structures, including the location of modified sites, their phylogenetic relations, and their general biosynthetic pathways have been extensively studied. The micellar structure of milk casein has also been extensively studied during the last 20 years (Davies et al., 1983; Horne and Dalgleish 1985; Holt 1985; Horne et al., 1989; Holt 1992), but, even if the existence of casein micelles is generally accepted, the existence of different subclasses and the relation between these submicelles is more difficult to establish (Holt and Horne, 1996). Surprisingly, little attention has been devoted to the real casein-phosphorylating enzyme, which has neither been purified to homogeneity nor cloned to date. The first enzyme able to phosphorylate casein was described in the pioneering work of Burnett and Kennedy (1954). Unfortunately, neither of the casein kinases, CK1 and CK2 (defined in this work), nor protein kinase CK2 was the physiological casein kinase. Casein phosphorylation is a posttranslational process (Bingham et al., 1972). Different authors have given extensive descriptions of partially purified enzymes capable of phosphorylating caseins, deriving either from the Golgi apparatus (Bingham and Farrell 1974; Lasa et al., 1997a), from mammary gland tissue (Bingham and Farrell 1974), more recently from fresh milk (Duncan et al., 2000). Until now, casein kinase from the lactating guinea pig mammary gland was the only type to be described as being isolated in pure form (Moore et al., 1985), even if the validity of the study was recently questioned (Duncan et al., 2000).

Protein kinase CK2 is a protein kinase able to phosphorylate milk proteins either in isolated form, or as micelles from native phosphocaseinate (Pitois et al., 1999). The phosphorylation of this latter substrate mod-

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ified some of its functional properties, and led to further understanding of its structure (Pitois et al., 1999). In order to better understand how a protein kinase modifies substrates with micellar structure, we have studied the action of protein kinase CK2 on fresh pasteurized skimmed milk. We describe in this work the action of protein kinase CK2 on milk and on different classes of micelles. In the course of the research, we found that a significant amount of endogenous casein kinase activity existed in the different micelle classes. We did not try to purify this enzyme, but defined its specificity towards caseins in its natural environment. We also analyzed the different micelle classes using different techniques [capillary zone electrophoresis (CZE), light scattering], as well as determining the mineral content of the different milk fractions used in this study. The intrinsic protein kinase does not exhibit a preference for a particular class of micelles, even if the structural differences among the different micelle classes are important.

MATERIALS AND METHODS

Milk, Enzymes, Substrates

Fresh milk was obtained from the institute's farm. The milk was skimmed, and pasteurized at 78°C for 12 s. Recombinant protein kinase CK2 from the yeast *S. pombe* was bacterially expressed and purified and assayed as already described (Pitois et al., 1999). Dephosphorylated caseins and other casein standards were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France), as was NaN_3 . They were of the same quality as proteins used in earlier works (Pitois et al., 1999). The reagents employed to prepare the buffers were of electrophoresis grade dissolved in Ultrapure water (Milli-Q quality). Urea and $[\gamma^{32}\text{P}]\text{ATP}$ were from Amersham Pharmacia Biotech Europe GmbH (Saclay, France).

Phosphorylation Assays

The protein phosphorylation assay with $\text{rspCK2}\alpha$ was the same as already described (Pitois et al., 1999), using purified $\text{rspCK2}\alpha$. Incorporation of P into substrates was estimated after TCA precipitation of the reaction mixture, and subsequent measurement of the radioactivity of the protein using a liquid scintillation counter (Tricarb 1500, Packard Instrument S.A., Rungis, France), as already described (Pitois et al., 1999). Milk ultrafiltrate (see below) was used as a physiological buffer during the phosphorylation of milk proteins by the endogenous milk kinase.

Milk Fractionation

Milk ultrafiltrate was obtained by the ultrafiltration at 4°C of skimmed pasteurized milk using a Diaflo filtration unit (Millipore-Amicon Bioseparations, Molsheim, France) equipped with a 10,000 Da ultrafiltration membrane, under a stream of nitrogen. Aliquots of 1 ml were frozen and stored at -20°C till further use. The isolation of micellar fractions was performed as described by Robson et al. (1985). A whole micellar fraction was isolated from 12 ml of fresh pasteurized skimmed milk by centrifugation at 20°C and 77,000 *g* for 2 h. Sub-fractions of the total micellar population were prepared by fractionating 12 ml of skimmed pasteurized milk by successive centrifugation steps at increasing speed. Each step lasted 15 min, and the temperature was 20°C during the whole experiment. The first centrifugation was at 7700 *g*, and the supernatant (Fraction S1) and the micellar pellet (Fraction C1) were collected. Fraction S1 was subsequently centrifuged at 12,200 *g*, to give supernatant S2 and pellet C2. Successive centrifugation steps at 17,200, 27,000, 38,500 and 73,400 *g* gave supernatants S3-S6 and pellets C3-C6. They were generally used immediately following their isolation unless otherwise specified in the text. The pellets were resuspended and diluted as required for further experiments either in buffer or in milk ultrafiltrate. Unless specified in the text, NaN_3 (0.01% w/v) was added to the buffer and milk ultrafiltrate used for the dilutions, to prevent bacterial growth.

Particle Sizing

Freshly prepared micelles, or milk, were diluted into milk ultrafiltrate, and filtered through a 0.45 μm Amicon filter directly into a 1-cm light path glass cuvet. Measurements of particle size were performed in a Coulter Model N4 submicron particle analyzer (Coulter Electronics Hialeah, FL). We used generally the equivalent of 20 μl of milk in 2 ml ultrafiltrate for the measurement. Results are given as a mean of 6 to 8 measurements.

Electrophoreses

Capillary zone electrophoresis. To dissociate the caseins, all samples (fresh skimmed pasteurized milk and submicelles) were dissolved in a 5 mM NaH_2PO_4 sample buffer containing 6 *M* urea and 5 mM dithiothreitol at pH 8 and left at least 1 h at room temperature before filtration (0.22 μm) and CZE analysis.

The electrolyte solution consisted of 10 mM NaH_2PO_4 , 6 *M* urea and 0.5% (w/v) methylhydroxyethylcellulose (MHEC) and the pH was adjusted to 2.5 with 4 *M* phosphoric acid. Before preparing the electro-

lyte, the urea solution at a concentration of 10 *M* was passed over a mixed-bed ion exchanger (AG 501-X8, Bio-Rad) to remove isocyanate and other ionic impurities. Before use, electrophoresis buffer was filtered through a 0.22 μm Millex-GS filter (cellulose esters, Millipore-Amicon Bioseparations).

CZE experiments were carried out on a Beckman P/ACE System 5000 (Beckman Coulter, Fullerton, CA) controlled by its software data system (P/ACE Station Version 1.0). The separations were performed using a hydrophilically-coated fused-silica capillary column CElect P1 (Supelco, Bellefonte, PA), 570 mm $\times$ 50 μm I.D., with a slit opening of 100 $\times$ 800 μm . The distance between detection window and outlet was 70 mm, resulting in an effective capillary length of 500 mm. The separations were conducted at 45°C with a voltage maintained at 30 kV grounded at the detector side providing a current of approximately 75 μA . Sample solutions were hydrodynamically injected at the anodic end for 10 s at 3.4 kPa. The eluted proteins were detected by absorption at 214 nm (data collection rate 5 Hz). Before each separation the capillary was flushed with 10 mM H_3PO_4 , H_2O and running buffer for 5 min each. The first electrophoretogram in a series was always discarded. Peaks were identified by comparison with authentic samples of individual proteins. Co-injections of the milk sample with standard κ -casein in an appropriate ratio were performed to obtain unambiguous identification of the peaks. The percentages of caseins were calculated by using peaks area as measured by electrophoresis corrected with migration time and response factor, which is different for each milk protein.

Urea-PAGE electrophoresis. These electrophoreses were performed on homogenous 20% Phast gels (Pharmacia) as described in Van Hekken et al. (1992) and in Pitois et al. (1999). Gels were stained using Coomassie blue and dried.

Autoradiograms

The gel was exposed overnight to a Phosphor Screen film (Kodak). It was subsequently scanned with a Storm 620 device (Molecular Dynamics, Amersham Pharmacia Biotech). The amounts of ^{32}P incorporated into the different milk proteins were estimated using the surface scanning routine of the ImageQuant software (Molecular Dynamics, Amersham Pharmacia Biotech).

Mineral Analyses

Samples of milk, ultrafiltrate and submicellar fractions were ashed at 550°C for 5 h in silica crucibles in duplicate or triplicate. The ashes were dissolved in 2 ml of 6 *M* HCl and diluted with deionized water before

the determination of Ca and Mg by atomic absorption spectroscopy (Perkin Elmer 1100 B) using an air-acetylene flame and wavelengths of 422.7 and 284.8 nm respectively. The data are the mean of three measurements.

Phosphorus was determined with malachite green reagent according to Eckman and Jäger (1993) after 15 min hydrolysis at 105°C with 2 *N* NaOH. Each measurement was duplicated.

RESULTS

Phosphorylation of Milk and Total Micelles by rspCK2 α

Using rspCK2 α , we could incorporate in 50 μl of milk (diluted to a 1 mg/ml casein concentration) an average of 540 pmol of P in 24 h (i.e. 0.26 P/mol casein) (Figure 1a). The total micellar fraction was also a substrate for rspCK2 α . The supernatant obtained by centrifugation of milk at 77,000 *g* was also phosphorylated by rspCK2 α . Up to 282 pmol of P were incorporated into 50 μg of proteins in the supernatant. Because the supernatant contains mainly whey proteins (61%) after the removal of the casein micellar fraction, it appears that the kinase is active towards these proteins as well. The proteins phosphorylated by rspCK2 α in milk and the total micellar fraction were identified on the autoradiogram of the urea-PAGE gel of the aliquot fractions of the reaction mixture withdrawn after different times. As can be seen from Figure 1b, in milk and the total micellar fraction, both α_s and β caseins are substrates for rspCK2 α . The reactivity of both types of casein is similar in the milk fraction, but, in the total micelles, α_s caseins are clearly the best substrate. In all of the samples, an unidentified peak, X, was also phosphorylated by rspCK2 α , with the extent of phosphorylation increasing with reaction time. In control experiments performed in the absence of added rspCK2 α , we measured the level of phosphate incorporated in different milk fractions after 5 h of reaction. We found a background incorporation of 9% for milk, of 15% for total micelles, and 26% for the supernatant, relative to the levels of incorporation of phosphate found in the presence of rspCK2 α . This demonstrated the existence of an endogenous protein kinase. To investigate this further, we studied the phosphorylation of the different micellar fractions in the absence of any added exogenous protein kinase.

Phosphorylation of Different Micellar Fractions by an Endogenous Protein Kinase

To better characterize the autophosphorylation of milk proteins, we prepared different fractions of mi-

celles, and incubated them in milk ultrafiltrate with [γ - ^{32}P] ATP under various experimental conditions. To avoid any undesirable proteolysis arising from the growth of microorganisms, we added NaN_3 to our reaction mixtures.

We checked the yield of phosphate incorporation into the proteins as function of time, age of the micelle preparation, and also divalent metal concentration. The Mg^{2+} and Ca^{2+} concentrations present in the assay of the endogenous kinase, carried out in milk ultrafiltrate in the absence of any added metal, were 1.5 mM and 0.75 mM respectively. In figure 2a, we present typical time dependent phosphate incorporation occurring in C3 micelles in the presence or in the absence of different combination of divalent metal. In the absence of any added metal, 87 pmol phosphate were incorporated in the micelles in 2 h (Curve 1). The addition of 15 mM MgCl_2 , of 15 mM MnCl_2 or of 15 mM MgCl_2 and 15 mM MnCl_2 in the reaction mixture led to a significant decrease of phosphate incorporation (less than 60 pmol) (curves 2, 3 and 4). When 15 mM CaCl_2 was added in the reaction mixture, 190 pmol of phosphate were incorporated into proteins within 2 h (Curve 5).

Typical phosphate incorporation yield into freshly prepared micelles and milk observed after overnight incorporation (17 h), in the presence or in the absence of added MgCl_2 or CaCl_2 in the assay are presented in figure 2b. C6 and C3 micelles could respectively incorporate up to 100 and 120 pmol phosphate in the presence of 15 mM added MgCl_2 , and up to 200 and 270 pmol phosphate in its absence. After 17 h, in the presence of 15 mM CaCl_2 incorporation yield reached 470 pmol phosphate. However, after storage for 1 d at 4°C as centrifugation pellets, the C3 and C6 micelles could only incorporate in a time dependent manner 82 and 58 pmol phosphate respectively in the absence of any added MgCl_2 (data not shown). The activity of the endogenous kinase showed little sensitivity toward heparin, in contrast to the high heparin sensitivity of the $\text{rspCK2}\alpha$ kinase (Hataway et al., 1980). Activity in C6 micelles was hardly affected in the presence of 1 $\mu\text{g}/\text{ml}$ heparin in the assay. In the presence of 40 $\mu\text{g}/\text{ml}$ heparin, phosphate incorporation was only reduced by 50% (data not shown). Figure 2c shows the result of a typical experiment designed to analyze the protein phosphorylation pattern. The autoradiogram revealed that α_s casein was phosphorylated to a greater extent than β casein in the absence of added $\text{rspCK2}\alpha$. This is similar to the results obtained when the total micellar fraction was phosphorylated by $\text{rspCK2}\alpha$, but in contrast to the phosphorylation of milk by the recombinant kinase. On the other hand, there was no evidence of the unknown material shown as X in Figure 1, which may suggest

that the endogenous kinases and $\text{rspCK2}\alpha$ have different specificities.

Determination of the Hydrodynamic Diameter of the Micelles by Laser Light Scattering

We measured the hydrodynamic diameter of the different micellar fractions by laser light scattering. Results are presented in Figure 3. The hydrodynamic diameter of the micelles decreased with the increasing centrifugal force used to prepare the different fractions. The size of the different micelles obtained in our study ranged from 247 ± 4.09 nm (C1 micelles) to 121 ± 3.9 nm (C6 micelles). Milk mean micellar diameter was 210 ± 2.25 nm.

Composition of the Different Micelles by CZE

Milk and micellar fractions. The content of different caseins was determined by CZE, for fresh skimmed pasteurized milk and the micellar fractions. A good separation of caseins was obtained using the method developed by De Jong et al. (1993) on a hydrophilically coated capillary at low pH. The peak identification was made according De Jong et al. (1993) and Otte et al. (1997) and was confirmed by analysis of individual casein standards (Figure 4). The α_{s2} casein (α_{s2} CN) appeared as several peaks migrating between 14.3 and 15.3 min, which is consistent with the existence of the different phosphorylation states of this protein. The α_{s1} casein (α_{s1} CN) migrated as two peaks at 15.7 min and 16.4 min, corresponding to the two differently phosphorylated α_{s1} CN-8P and α_{s1} CN-9P (namely α_{s0} CN) proteins respectively. The milk used was a bulk milk containing several genetic variants. The major β casein peaks coincided with β casein A1 (β CN A1, 18.7 min) and β casein A2 (β CN A2, 19.5 min) and the minor peak (17.9 min) contained the B and C variants. The κ casein contained the genetic variants, A and B, with two major peaks at 17.5 and 18 min, flanking the β CN B/C peak. It has to be noted as reported elsewhere that κ casein response is rather low compared to the response of other caseins. The repeatability of the electrophoresis separation system was assessed by analyzing consecutive injections of milk samples. The relative standard deviation values (RSDs) of the migration times of caseins were less than 0.3%. The day-to-day variation was estimated by analysis of the model sample at intervals over a 4-month period, which gave RSD values for the migration times of less than 0.8%. The average casein composition of skimmed pasteurized milk is illustrated in Table 1. The contents of α_s casein, β casein and κ casein were also determined in the micellar fractions obtained by successive centrifugations (Figure

Figure 1. Phosphorylation of proteins from pasteurized skimmed milk and total micelles by *rspCK2 α* , and identification of the labeled proteins.

a. Phosphorylation of proteins from pasteurized skimmed milk and total micelles by *rspCK2 α* . Pasteurized skimmed milk (▲), the total micellar fraction (△), and the resulting supernatant after centrifugation at $77,000 \times g$ (●) were reacted with $0.865 \mu\text{g}$ *rspCK2 α* (specific activity $26,000 \text{ pmol/min/mg}$ using dephosphorylated caseins as substrate) in a $50 \mu\text{l}$ final volume (protein concentration in the assay 1 mg/ml). Buffer was 150 mM TEA , at 30°C , in the presence of 15 mM MgCl_2 and $250 \mu\text{M ATP}$. Phosphate incorporation was measured as described in the text.

b. Identification of the proteins from pasteurized skimmed milk (1) and total micellar fraction (2) phosphorylated by *rspCK2 α* .

Aliquots from the phosphorylation mixture were run on urea-PAGE. The gels were stained and exposed to Phosphor Screen. Reaction plots are shown for α_s casein (▲), β casein (●) and the unidentified peak (▼).

5). It is clear that the content of κ casein increased as the micellar size decreased. Proportion of κ casein in the smallest micelles was two to three times greater than in the largest ones.

73,400 $\times g$ Supernatant and Milk Ultrafiltrate

The supernatant centrifuged at $73,400 \times g$ contained 4 to 5 times less milk proteins (caseins + whey proteins) than milk or micelle fractions. It contained, as judged from CZE electrophoregram analysis, mainly whey proteins (61%). It also contained β casein (23%), κ casein (6%) and only 10% α_s caseins. Milk ultrafiltrate did not contain any detectable casein or whey proteins.

Mineral Composition of Micellar Fractions

The changes in casein composition of the micellar fractions were accompanied by some changes in their

contents of calcium, magnesium and phosphorus (Table 2). The content of calcium appeared to decrease as the micellar size decreased whereas the magnesium content remained essentially constant. The content of phosphorus was also rather constant except in the case of the smallest micelles where it was low. The result of these data was a constant ratio of calcium to phosphorus.

DISCUSSION

RspCK2 α is able to phosphorylate milk proteins in a native environment, consisting either of skimmed pasteurized milk, or of total micellar fraction. The phosphorylation yields measured here are lower than those determined by Pitois et al. (1999) with phosphocaseinate (840 pmol P in $50 \mu\text{g}$ proteins). Pitois et al. (1999) found most of the label in β casein, which is in

Figure 2. Phosphorylation of milk proteins by endogenous protein kinase.

a. Time course of incorporation of phosphate into freshly prepared micelles of fraction C3. The fractions were incubated in milk ultrafiltrate at a 1 mg/ml final concentration of protein containing 250 μ M ATP, without addition of metal (curve 1, bold line, ●), with addition of 15 mM MgCl_2 (curve 3, thin line, ▽), 15 mM MnCl_2 (curve 4, dashed line, △), 15 mM MgCl_2 + 15 mM MnCl_2 (curve 2, thin line, ◇), or 15 mM CaCl_2 (curve 5, thin line, ■).

b. Incorporation of phosphate into freshly prepared micelles of fractions C3 and C6, and skimmed milk. The fractions were incubated in milk ultrafiltrate at a 1 mg/ml final concentration of protein containing 250 μ M ATP, with (+) or without (-) the addition of 15 mM MgCl_2 or 15 mM CaCl_2 . Reaction time was 17 h.

c. Identification of the proteins from C6 micelles which were phosphorylated by endogenous protein kinase after 17 h reaction without addition of MgCl_2 .

agreement with the good substrate capacity of isolated β casein for $\text{rspCK2}\alpha$. In our present study α_s casein was preferentially labeled by $\text{rspCK2}\alpha$ or was at least as good a substrate as β casein for this kinase. This result could be explained by a higher accessibility of β casein in PPCN than in milk or micelles. This may be a result of the different materials used: PPCN is prepared by microfiltration and diafiltration of milk, which removes some of the calcium phosphate from the casein micelles (Schuck et al., 1994), leaving them perhaps more liable to structural change. In the present study, we used fresh pasteurized milk or fractions derived from it.

The control experiment performed without addition of the $\text{rspCK2}\alpha$ kinase in milk, in micellar fractions, and also in supernatant revealed the presence of overphosphorylated proteins. The phosphate incorporation into proteins was time dependent. Spontaneous phos-

phate incorporation represented up to 26% of the total phosphate incorporated in the presence of $\text{rspCK2}\alpha$. The addition of Mg^{2+} , of Mn^{2+} or of Mg^{2+} and Mn^{2+} led to significant inhibition of phosphate incorporation. Phosphate incorporation was stimulated by Ca^{2+} . The phosphate was incorporated covalently into the caseins, as deduced from urea-PAGE and autoradiograms. We attributed this incorporation to an endogenous kinase, with properties different from those of $\text{rspCK2}\alpha$. This

Table 1. Relative proportions of milk caseins determined by capillary zone electrophoresis (N = 19)¹

	α_{s2} casein	α_{s1} casein + α_{s0} casein	κ casein	β casein
%	8.99	44.03	7.25	39.73
SD	2.52	3.71	3.51	2.78

¹ whole casein was considered to be 100%.

Table 2. Mineral composition of micellar fractions.

Micellar fraction	Content (mg /g dry matter)			Ca/P
	calcium	magnesium	phosphorus	
C1	19.9	1.57	10.1	1.97
C2	17.4	1.49	10.9	1.59
C3	17.6	1.72	9.2	1.91
C4	17.2	1.84	10.5	1.64
C5	16.5	1.57	10.2	1.62
C6	14.1	1.48	8.1	1.74

Relative standard deviations for calcium, magnesium and phosphorus determination of the same sample were respectively 0.6, 1.1 and 1.2% and near 5% for duplicate or triplicate sampling.

casein kinase proved to be relatively unstable, since 24 h after the micelles had been prepared, almost 70 percent of the activity had been lost and the remaining activity was almost insensitive towards added Mg^{2+} . As the proteins were stored as pellet at 4°C, which excludes micelle dissociation, the loss of activity of the kinase may be attributed to its intrinsic instability, or by proteolysis by plasmin. In milk, the activity of the enzyme was lower, possibly because of the age of the milk when the assay was made. This enzyme, found in all micellar fractions, exhibited a preference for α_s caseins. The endogenous casein kinase is different from the well known protein kinase CK2, as it is inhibited only by relatively high heparin concentrations, it is active in the presence of relatively low Mg^{2+} concentration, and inhibited by

Figure 3. Hydrodynamic diameter determination by laser light scattering, after dilution of micelles in milk ultrafiltrate. **Figure 4.** Protein content of fresh pasteurized skimmed milk and micelles analyzed by capillary zone electrophoresis (a: milk; b: C3 pellet; c: C6 pellet).

the addition of either 15 mM Mg^{2+} in the assay, 15 mM Mn^{2+} or 15 mM Mn^{2+} + 15 mM Mg^{2+} . Protein kinase CK2 is characteristically inhibited by 1 μM heparin, is activated by Mg^{2+} and Mn^{2+} (Hataway et al., 1980, Chardot et al., 1995), and is unable to use Ca^{2+} as a metallic cofactor. The presence of significant kinase activity in the 73,400 $\times g$ supernatant, containing mostly whey protein after the removal of casein indicates a broader specificity of the milk kinase, and confirms that the kinase is not associated with a particular milk sub-fraction.

The endogenous kinases which have already been described are obviously different from protein kinase CK2 (Duncan et al., 2000, Brunatti et al., 2000). The comparison of the properties of the different enzymes which have been claimed to be the true physiological casein kinase is very difficult, especially because of the different assay methods which have been used in different laboratories. These enzymes exhibit different specificity, especially with respect to the catalytic metal used in the assay. Our results confirm the importance of Ca^{2+} in endogenous kinases activity as already pointed out by Szymanski and Farrell (1982), but contrast with results of other groups. Bingham (1979) reported requirement of the enzyme for divalent metal, with preference for Mn^{2+} . In further work, Bingham et al. (1988) found Ca^{2+} most effective for α lactalbumin phosphorylation, and Mn^{2+} for β casein. Together with the observation that milk kinase sensitivity towards Mg^{2+} decreased with time, this may lead to the speculation that two or more protein kinases may participate in casein phosphorylation in vivo.

The casein content of the milk did not vary over the period of this research (5 mo) because the herd did not

Figure 5. Casein contents are given as percentage of milk total caseins.

have a phased calving pattern. The relative proportions of major caseins in milk are comparable with the results of Davies and Law (1977), Donnelly et al. (1984) and

Vallejo-Cordoba (1997) who performed a quantification of bovine milk caseins using respectively permeation chromatography, ion exchange chromatography or capillary electrophoresis. It is clear that the content of κ casein increased as the micellar size decreased. This result is very similar to that reported by Donnelly et al. (1984) and Dalgleish et al. (1989) and is compatible with the general hypothesis that κ casein is preferentially localized in the micelle surface. On the other hand, in our experiments, the increase in the content of κ casein was not balanced by a large decrease in the content of other caseins. It is seen that there is a slight increase in the proportion of the α_s caseins as the micellar size increases, and a slight decrease of the proportion of β casein, which suggests that there is some preference for β casein to be near the surface of the micelles. This is in agreement with the results of Davis et al. (1977), Donnelly et al. (1984) and Diaz et al. (1996), but not with those of Dalgleish et al. (1989). All of these studies used different methods of analyzing the composition of micelles and their surface, so it is still somewhat unclear which of the α_s or β casein has the higher concentration at the micellar surface. On the other hand, calcium concentration decreases with micellar size, and the ratio of Ca to P was rather constant. This result suggests that the involvement of calcium phosphate in micelle formation and structure is the same in the different milk micelles.

We saw no evidence that the endogenous kinase was in different concentration in the different micellar size classes. Figure 2 shows that the difference between pellets C3 and C6 is not significant. Moreover, endogenous protein kinase activity was observed in the supernatant of the total micellar fraction. There is no evidence, therefore, that the kinase is bound to the surface of the casein micelles.

The fact that the endogenous kinase exists in milk is established, but that there are few reports of superphosphorylated caseins in natural milk, suggests that it is the lack of ATP in the milk which prevents this from occurring. Of the caseins, it is only κ casein which has been reported to have a rather variable phosphorylation pattern (Vreeman et al., 1977). The continued presence of the active kinase after pasteurization suggest that it is relatively heat-stable, although the fact that it is bound to one of its substrates (the caseins) may help to protect it from heat denaturation.

CONCLUSION

A protein kinase activity has been found in milk, and in different milk micellar fractions. Milk contained sufficient amount of Mg^{2+} and Ca^{2+} for this enzyme to phosphorylate caseins if ATP was added to the reaction

mixture. This protein kinase is exhibited a time dependent action on milk proteins. This enzyme was certainly not CK1 or CK2, especially due to its lack of sensitivity toward low heparin concentrations, and to its capacity to be activated by Ca^{2+} . The protein kinase found in milk did not exhibit a particular reactivity with the different classes of micelles, even if their size, and composition were rather different. Its best substrate was α_s CN.

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