Overphosphorylation of Milk Caseins by a Recombinant Protein Kinase CK2 Catalytic Subunit

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Milk caseins have been phosphorylated by a recombinant protein kinase CK2 catalytic subunit from *Schizosaccharomyces pombe* (rspCK2α). Phosphate incorporation stoechiometries into purified caseins and into native phosphocaseinate, a substrate exhibiting a micellar-like structure, were determined. We incorporated 2.01 mol of P/mol of α-casein, 6.46 mol of P/mol of *β*-casein, up to 0.29 mol of P/mol of *κ*-casein in 4 h, and more than 1.36 mol of P/mol of casein into phosphocaseinate under optimized conditions. Phosphocaseinate was sequentially phosphorylated; *β*-caseins being labeled at first; α-caseins being labeled later; and to a lower extend, *κ*-caseins were the last to be phosphorylated. The solubility of phosphocaseinate micelles increased by 1.34 from 65 to 87%, and its rennetting time was increased 2.88 times. These results are discussed in terms of plausible structural organization of caseins micelles and the effect of phosphorylation on their structure.

Keywords: *CKII; protein kinase CK2; phosphorylation; caseins; micelles*

INTRODUCTION

The phosphorylation state of caseins and their micellar organization are essential to explain their cheesemaking capacity. Addition or removal of phosphate is an efficient method for modifying the functional properties of proteins [see Matheis and Whitaker (1984) and Chardot et al. (1998a) for reviews]. Chemical phosphorylation of proteins, a method used for many years, improved their functional properties (Matheis and Whitaker, 1984; Popineau et al., 1995; Medina et al., 1996). Chemically overphosphorylated caseins were less sensitive toward proteolysis by pepsin (Medina et al., 1996).

Major limitations to the use of chemicals, especially as phosphorylating agents, are the toxicity of the reagents and the occurrence of side reactions such as aggregations. This is why enzymes have been used in recent studies to phosphorylate proteins. From the study of the sequences of different legume proteins, it was found that not all phosphorylatable target amino acids were phosphorylated in vitro (Chardot et al., 1998b). Enzymatically phosphorylated legume proteins exhibited new functional properties with respect to the nonphosphorylated ones (Ross and Bathnagar, 1989a,b; Seguro and Motoki, 1989; Campbell et al., 1992; Aluko and Yada, 1995; Ralet et al., 1996; Chardot et al., 1998b). Chemically or enzymatically dephosphorylated caseins have been used as model substrates for the study of the kinetic properties of protein kinases for a long time (Walsh et al., 1968), but little work has been done on the enzymatic phosphorylation of individual caseins (Hataway and Traugh, 1984; Yoshikawa et al., 1989), especially to achieve stoichiometric phosphate incorporation (Chardot et al., 1998b). The sequence and the phosphorylation sites of caseins have already been determined (Mercier, 1981). The phosphorylation of caseins is occurring posttranslationally (Bingham et al., 1972; Bingham and Farrel, 1974; Bingham and Groves, 1979), and not all the phosphorylatable residues are phosphorylated in vivo.

In this paper, we describe the expression and the purification of a bacterially expressed protein kinase CK2 catalytic subunit from the yeast *Schizosaccharomyces pombe* (rspCK2α). This enzyme has been used to overphosphorylate milk proteins, isolated or in a micellar state (phosphocaseinate). We have determined and compared the kinetic parameters and incorporation stoichiometries for isolated and micellar caseins. We also have studied the relation between phosphorylation state and solubility of a model substrate, phosphocaseinate. Finally, we have studied the influence of overphosphorylation of phosphocaseinate toward hydrolysis by chymosin.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP was from Amersham (Orsay, France). Dephosphorylated caseins (whole caseins, at least 80% dephosphorylated) were from Sigma (Ref C 4032) (L'Isle d'Abeau, France), as were α- (ref C6780), *β*- (ref C6905), and *κ*-caseins (ref C0406). Phosphocaseinate was obtained from Dr. Maubois (Pierre et al., 1992). Rennet was from Chris-Hanssen (Arpajon, France). Phosphocellulose was from Whatman Scientific Ltd (United Kingdom). Hitrap heparin and Resource S chromatographic column were from Pharmacia Biotech (Orsay, France). Leupeptin, pepstatin, and Pefablock were purchased from Boehringer Mannheim (Meylan, France). Novex ready-to-use gels were obtained from Poly Labo (Strasbourg, France). Ampicilin and IPTG (isopropylthio-*β*-D-galactoside) were from Promega (Charbonnière, France). Scintillation liquid (Ecolite) was from ICN Biomedical (Orsay, France). Gibco growth media were obtained from Life Technology (France). All other chemicals were of the best available quality. Expression vector pGB1containing the sequence corresponding to

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S. pombe protein kinase CK2 catalytic subunit was a generous gift from I. Roussou, (Roussou and Draetta, 1994). *Escherichia coli* BL21 strain was obtained from Promega (Charbonnière, France).

Methods. *Expression of rspCK2α in E. coli.* BL21 D lys *E. coli* cells were transformed by the expression vector pGB1 containing the sequence coding for the *Sp*CK2α subunit. The cell culture was performed as described in Benetti et al. (1997).

Protein Extraction. Proteins from 300 mL of induced bacterial culture were extracted as described in Benetti et al. (1997) and used in the subsequent purification steps.

Purification of rspCK2α. The supernatant from the lyzed bacteria (corresponding 300 mL of bacterial culture) was loaded onto a P11 phosphocellulose gel (10 mL), equilibrated with 25 mM Tris-HCl buffer, pH 8, containing 0.1 mM PMSF, 0.5 mM DTT, and 20% glycerol. The enzyme was eluted by a NaCl gradient in the same buffer from 0.2 to 1 M (total volume, 60 mL). Active fractions were pooled and concentrated with a Diaflo (Amicon) concentration device equipped with a 10-kDa membrane under a stream of nitrogen at 4 °C. The enzymatic extract was then submitted to Resource S chromatography.

After equilibration with 25 mM TEA buffer containing 100 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, and 10% glycerol, pH 8, the active fractions were loaded onto a Resource S column (1 mL) equilibrated in the same buffer. The column was rinsed with 3 mL of equilibration buffer and then eluted using a 0.1−0.6 M NaCl gradient (10 mL). Fractions (0.5 mL) were collected, assayed for activity, and stored at −20 °C till use.

Substrates for rspCK2α. Caseins were dissolved in water, and the pH was adjusted to 6.8 with diluted NaOH. In the case of phosphocaseinate, more vigorous conditions were used to obtained a good solubilization. After dispersing the powder into water, the solution was heated at 50 °C and mixed with a Waring blender for 5 min using the high speed. The pH was then adjusted to 6.8 using diluted HCl. Phosphocaseinate was prepared daily, and solubilization was checked visually.

Protein Determination. Protein concentration was estimated using the Bio-Rad protein assay reagent using bovine serum albumin as a standard for protein rspCK2α. In the case of milk caseins, proteins were quantitated gravimetrically or by using the method of Lowry et al. (1951) using the Bio-Rad DC protein assay kit for the supernatant from ultracentrifugation experiments.

rspCK2α Kinetic Study. During its purification and characterization, rspCK2α activity was determined as already described (Chardot and Meunier, 1994). Briefly, the proteins were phosphorylated in the presence of [γ-^{32}P]ATP, and TCA precipitated. After the proper rinses, the radioactivity of the pellets was counted by liquid scintillation in a Packard liquid scintillation counter, using Ecolite scintillation liquid (ICN, Orsay, France). One unit is the amount of enzyme that incorporates 1 pmol of phosphate in 25 μg of dephosphorylated caseins (0.5 mg/mL) at 23 °C, in a 100 mM, pH 7.8, TEA buffer containing 100 mM NaCl, 10 mM $MgCl_2$, and 20 μM ATP. Specific radioactivity of ATP was determined upon counting of 5 μL of the reaction mixture. For the determination of the kinetic parameters with different substrates, assays were performed at 30 °C in a 100 mM, pH 7.8, TEA buffer containing 100 mM NaCl plus 10 mM $MgCl_2$, [γ-^{32}P]ATP (from 0.1 to 20 μM), and proteic substrates (from 0 to 3 mg/mL). Data were analyzed using the Lineweaver−Burk representation. Enzyme concentration was always close to 0.6 nm.

Overphosphorylation of Proteins. For determination of maximal P incorporation in different substrates, a reaction was performed at pH 6.8 and 30 °C for 4 h in the presence of 200 μM [γ-^{32}P]ATP, 15 mM $MgCl_2$, and β-casein 0.21 mg/mL, α-casein 0.23 mg/mL, κ-casein 0.5 mg/mL, or phosphocaseinate 0.08 mg/mL with the reaction volume being 50 μL. Phosphate incorporation level was determined by counting the radioactivity incorporated in the proteins upon TCA precipitation. In all experiments, the enzyme concentration was always kept close to 0.6 nM.

For the solubility and succeptibility to rennet studies of PPCN, overphosphorylation of proteins was performed at 30 °C for 3 h in a minimal reaction medium containing 200 μM cold ATP, 15 mM $MgCl_2$, and 1 mg/mL PPCN with the reaction volume being 50 mL. The pH of the reaction medium was checked and was always kept at pH 6.8. The phosphate incorporation level was determined by running small-scale parallel experiments with [γ-^{32}P]ATP with assay volume being 50 μL.

Milk Proteins Phosphate Content Determination. Phosphate content of isolated milk proteins was determined upon alkaline hydrolysis, as decribed by Queiroz et al. (1997) using the method of Ekman and Jager (1993).

Gel Electrophoresis and Autoradiograms. (a) SDS−PAGE of Bacterially Expressed Protein Kinase CK2 Catalytic Subunit. SDS−PAGE was carried out according to the method of Laemmli (1970) using a 4−20% ready-to-use polyacrylamide gels (Novex, San Diego) in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Gels were run under 120 V for 90 min. Gels were stained with Coomassie blue G-250 using the method of Neuhoff (1988).

(b) Urea Polyacrylamide Gel Electrophoreses of Milk Proteins. They were run according to Van Hekken et al. (1992). Briefly, Pharmacia ready-to-use 12% Phastgels were soaked 30 min in a 6.6 M freshly prepared urea solution containing 0.112 M Tris and 0.112 M sodium acetate with the pH being adjusted to 6.4 with acetic acid. The gel was left over 15 min. Samples were prepared in the same buffer plus 10% 2-mercaptoethanol and 0.025% bromophenol blue. Electrophoreses were run under 300 V, at 15 °C for 120 min, using native buffer strips (Pharmacia). Gels were stained using Coomassie blue staining and dried.

(c) Autoradiograms of Phosphorylated Phosphocaseinate. Dried urea polyacylamide electophoreses gels were exposed to Phosphor Screen film (Kodak, Sigma L'Isle D'Abeau France) (generally 2 h to overnight) and subsequently scanned with a Storm 620 device (Molecular Dynamics, Amersham Pharmacia Biotech, Orsay France). Autoradiograms were analyzed using the ImageQuant software (Molecular Dynamics, Amersham Pharmacia Biotech, Orsay France). The relative amount of ^{32}P incorporated into proteins was estimated from peak height.

Solubility. Phosphocaseinate solution (1 mg/mL, 50 mL total volume) was phosphorylated for 3 h at 30 °C as described in the overphosphorylation of proteins section and then spun at 77000*g* at 20 °C in a Kontron ultracentrifuge (Famelard et al., 1996). The supernatant

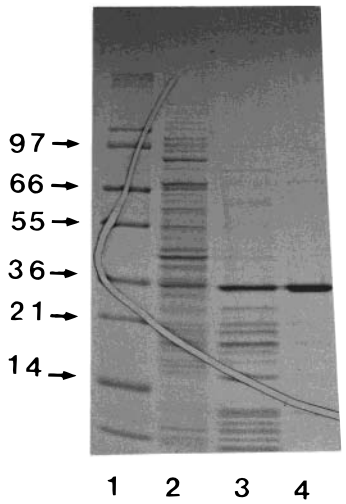

Figure 1. Purification of rspCK2α. SDS−PAGE was performed as described in Experimental Procedures. Lane 1, molecular weight markers; lane 2, crude extract (6.3 μg of proteins); lane 3, active fractions from phosphocellulose chromatographic step (pool) (1 μg); lane 4, active fractions from Resource S column (pool) (1.8 μg).

Table 1. Purification of Recombinant rspCK2α[a]

	vol (mL)	total proteins (μg)	total activity (U)	specific activity (U/mg)	purification	yield
15000*g* supernatant	25	7 875	25 782	3 274	1	100
P cellulose	12	480	14 940	31 126	9.50	57.9
Resource S	2	70	2 802	40 024	12.22	10.8

[a] The values correspond to experiment performed with 150 mL of bacterial culture as starting material. After the phosphocellulose step, the active fraction pool was submitted to Resource S chromatography.

was collected, and the protein concentration was estimated by the method of Lowry et al. (1951) using the Bio-Rad DC protein assay kit.

Study of the Effect of Phosphorylation on Coagulation Time by Rennet. Phosphocaseinate solution (1 mg/mL, 50 mL total volume) was phosphorylated for 3 h at 30 °C as described in the overphosphorylation of proteins section and then concentrated to 1 mL in a Amicon Diaflo (Millipore, St Quentin, France) using a 30-kDa membrane at 5.3 bar, 4 °C, under a stream of nitrogen. The pH of the sample was checked and adjusted to 6.6 with diluted NaOH if necessary. Rennet (10 μL of a 1/100 diluted solution) was added to a 200-μL sample, and coagulation was visually followed at 37 °C. Control experiments, lacking rspCK2α, were performed at the same time. Each experiment and control was performed twice on triplicate samples.

RESULTS

Expression of Recombinant rspCK2α. A 37-kDa peptide was expressed under the direction of the bacteriophage T7 promoter in recombinant *E. coli* BL21 cells. (Figure 1, lane 1). This was the expected size of rspCK2α. The expression of rspCK2α in *E. coli* appeared to be maximal about 1 h after induction with IPTG (data not shown).

Purification of rspCK2α. Table 1 describes the results of a typical purification procedure for the rspCK2α. Figure 1 checks the purity of the enzyme. rspCK2α expressed in *E. coli* has been purified using two chromatographic steps. Using phosphocellulose step and Resource S chromatography, we obtained 70 μg of protein with a specific activity of 40,024 U/mg from 150 mL of induced culture. The enzyme was homogeneous as judged from SDS−PAGE analysis (Figure 1, lane 3). The purified enzyme was losing its activity within hours, whatever the purification methods used. It was frozen and stored at −20 °C till use (at least during 1 month). Under these conditions, the enzyme is stable. All subsequent studies were performed with the enzyme purified using the Resource S chromatography.

Table 2. Maximal Phosphate Incorporations by rspCK2α into Caseins[a]

substrate	P content of cow's milk proteins	P content (this study)	theoretical P incorp by rspCK2α	max P incorp
α caseins	α-S1 8−9	6.64 ± 0.57	α-S1 +3	2.01
	α-S2 10		α-S2 +4	
β-caseins	4−5	3.67± 0.51	+4	6.46
κ-caseins	1	1.65	+2	0.29
phosphocaseinate[b]	ND[c]	43,760	ND	13,640

[a] All results are given in mol of phosphate/mol of protein or micelle. [b] ND, not determined. [c] One micelle = 10 000 casein molecules.

Determination of the Phosphate Content of Milk Purified Proteins. The results of phosphate content determination are given in Table 2. Using the green malachite method, we found a phosphate content of 3.67 P/mol in the case of commercial β-casein, α-casein contained 6.64 P/mol, and κ-casein contained 1.65 P/mol. Phosphate content of PPCN was 1600 mg/100 g.

Phosphorylation of Purified Milk Proteins. Phosphate incorporation with respect to casein concentration upon the action of rspCK2α was determined. Phosphorylation has been performed on individual caseins for 30 min (Figure 2A). As judged from the k_{cat}/K_m values given in Table 3, β-casein (6148 $s^{-1} \times \mu M^{-1}$) is the best substrate, followed by α-caseins (2606 $s^{-1} \times \mu M^{-1}$) with κ-caseins (723 $s^{-1} \times \mu M^{-1}$) being the poorest substrate.

The results of phosphate incorporation for 4 h at 15 mM Mg^{2+} and 200 μM ATP concentrations (30 °C) and at fixed casein concentrations are given in Table 2. Best substrates were β-caseins (6.46 P/mol) at 0.21 mg/mL in the assay, followed by α-caseins (2.01 P/mol) at 0.23 mg/mL in the assay; κ-casein could only incorporate 0.29 P/mol at 0.5 mg/mL in the assay. Protein kinase CK2 phosphorylates proteins on Ser, Thr and Tyr, in an S/T/Y−X-X−D/E environment (Kuenzel et al., 1987; Chardot et al., 1995; Wilson et al., 1997). We have calculated the number of sites that could be modified by spCK2α on the proteins used in this study, using the find patterns subroutine of the Wisconsin GCG Software (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI). Protein sequences were fetched from the Pir database (National Biomedical Research Foundation at Georgetown) and were compared with the protein kinases CK2 consensus sequences. Results are given in Table 2. In the case of αS1 casein (Pir reference: Kabosb), we found three consensus sequences for protein kinase CK2 not phosphorylated. Three phosphates could be added upon the action of protein kinase CK2. In the case of α-S2 casein (Kabos2), four phosphates could be added. κ-Casein (Pir reference: Kkbob) could bind two phosphates, and β-casein (Pir reference: Kbboa2) could gain four extra phosphates upon the action of protein kinase CK2.

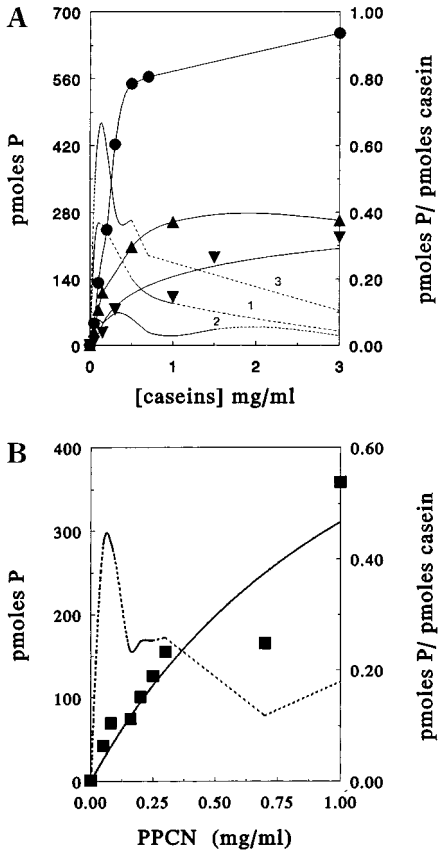

Figure 2. Phosphate incorporation into milk proteins. Purified proteins from milk or phosphocaseinate were incubated with purified recombinant rspCK2α for 30 min. Phosphorylation yield was calculated upon TCA precipitation and counting of the radioactivity incorporated in the pellets as described in Experimental Procedures. Left axis corresponds to total phosphate incorporation (solid lines). Right axis corresponds to the phosphate incorporation stoichiometry into proteins (dashed lines). (A) Purified proteins: α-caseins (▲ and dashed line 1), *κ* caseins (▼ and dashed line 2), *β* casein (● and dashed line 3). (B) Phosphocaseinate, (■) incorporation into phosphocaseinate (left axis); (− −) incorporation stoichiometry (right axis).

Table 3. Kinetic Properties of rspCK2α

substrate	K_{mapp} (μM)	k_c (s^{-1})	k_c/K_m ($s^{-1} \times \mu M^{-1}$)
ATP	5	1.74×10^{-2}	3480
α caseins	9.59	2.5×10^{-2}	2606
β-caseins	8.75	5.38×10^{-2}	6148
κ-caseins	26	1.88×10^{-2}	723
phosphocaseinate	0.159[a]	1.43×10^{-2}	ND

[a] In mg/mL. [b] ND, not determined.

Kinetic Measurements. The kinetic parameters of the purified rspCK2α have been determined (Table 3). k_c was 1.74×10^{-2} s^{-1}, K_m for ATP was 5 μM, and K_m for Mg^{2+} was 2.5 mM using phosphocaseinate as the substrate. Inhibition by heparin was checked and was 50% at 0.02 μg/mL (data not shown).

Phosphorylation of Phosphocaseinate. Phosphocaseinate is a substrate for rspCK2α. For protein concentrations higher than the optimal one (0.08 mg/mL), incorporation stoichiometries decreased (Figure 2B). Upon 4 h reaction, phosphocaseinate was prone to incorporate up to 13,640 phosphate molecules/mol of micelle (one micelle of phosphocaseinate consisting of 10,000 caseins). ^{32}P-labeled phosphocaseinate was submitted to urea electrophoresis and to autoradiography. In Figure 3A, we show urea PAGE performed with PPCN, phosphorylated by rspCK2α for different times (lanes 2−4). α- and *β*-caseins (commercial, lanes 5 and 6) are also shown, with the position of *κ*-casein being determined from other experiments (not shown). It can easily be observed that the commercial *β*-caseins (Figure 3A, lane 1) are exhibiting a very different electrophoretic behavior with respect to the *β*-casein from PPCN (Figure 3A, lane 6). α-Casein from PPCN was mainly composed of α_{S-1} caseins, as judged from SDS−PAGE (data not shown) The gel has been stained, dried, exposed to a Phosphor Screen, and scanned (Figure 3B). In Figure 3C, we have analyzed lane 2, corresponding to 15 min action of the enzyme on phosphocaseinate. The count presented on the *Y* axis is relative. The *X* axis corresponds to migration distances on the gel. From the results presented in Figure 3D, it appears that *β*-caseins were labeled first, followed by α-caseins. For very long times of phosphorylation, phosphate was also incorporated into *κ*-caseins. Some label was also found in the *x* peak. After Coomassie staining, no detectable protein was found in this peak. It was the ATP peak.

Effect of the Storage Temperature on the Micelle Substrate Capacity. The micellar PPCN has been stored at 4 or −20 °C. After 24 h, aliquot fractions were phosphorylated by rspCK2α at 30 °C. Results are given in Figure 4. The capacity of PPCN to be phosphorylated decreased when storage temperature decreased. Optimal conditions to get high phosphorylations yields were obtained with freshly prepared phosphocaseinate stored at 20 °C for less than 5 h.

Study of the Solubility of Phosphocaseinate as a Function of Its Phosphorylation State. In Table 4, we present the results of the study of the effect of phosphorylation on the solubility of phosphocaseinate. After 2 h of ultracentrifugation, we observed that the pellet of the overphosphorylated sample was becoming transparent with the control one remaining white (data not shown). The solubility of the overphosphorylated sample was increased from 65 (control value) to 87 (in mg of protein/100 mL) (Table 4).

Study of the Effect of Phosphorylation on Coagulation Time by Rennet. Under our conditions, coagulation of phosphocaseinate at 25 mg/mL occurred 117 ± 2 s after chymosin addition in the control experiment and 414 ± 2 s after chymosin addition in the case of the overphosphorylated sample.

DISCUSSION

Milk proteins, especially dephosphorylated caseins, are widely used as protein kinases substrates (Walsh, 1968), but little work has been published on the enzymatic phosphorylation of isolated milk proteins. A major limitation to the use of protein kinases as tools to modify proteins is the low amount of enzymes, especially protein kinase CK2, found in living organism (Benetti et al., 1997). Bacterially expressed proteins are an almost unlimited source of enzymes. From 150 mL of bacterial culture, we could purify up to 70 μg of an apparently homogeneous *S. pombe* recombinant protein kinase CK2 catalytic subunit.

Under our experimental conditions, a significant amount of phosphate was incorporated into purified caseins. The best substrate was *β*-casein (6.46 mol/mol), giving a maximal content of 10.13 P/mol, exceeding the maximal phosphate content expected upon the action of rspCK2α (8−9 P/mol). This can be attributed to the

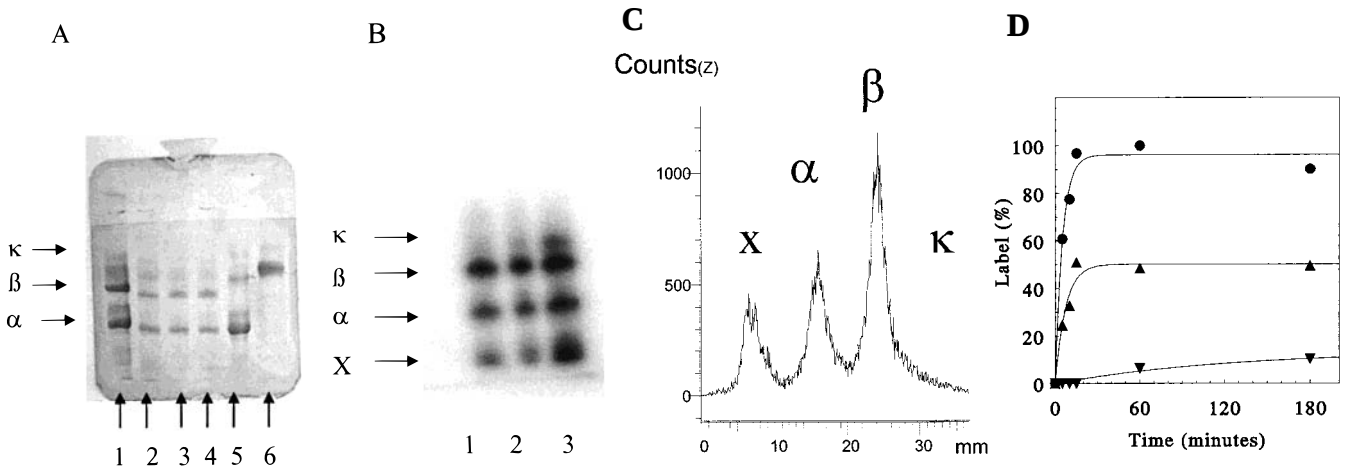

Figure 3. Kinetics of ^{32}P incorporation into the individual proteins from phosphocaseinate Phosphocaseinate (1 mg/mL) was incubated with purified rspCK2α at 30 °C as described in Experimental Procedures. After different times, sample were withdrawn and submitted to urea PAGE. The gels were dried and stained (panel A). Lane 1 corresponds to nonphosphorylated native PPCN, lane 2 corresponds to PPCN phosphorylated 15 min, lane 3 corresponds to PPCN phosphorylated 1 h, and lane 4 corresponds to PPCN phosphorylated 3 h. Lanes 5 and 6 correspond to commercial α- and *β*-caseins, respectively. The gel was exposed to Phosphor Screen (panel B). Lanes 1−3 correspond to 15 min, 1 h, and 3 h of phosphorylation, respectively, and individual lanes were analyzed. Analysis of lane 1 is presented in panel C. Protein peaks were identified with respect to standards, and the label incorporated into each protein was then plotted versus time (panel D). The incorporation value into *β*-casein was arbitrarily taken as 100%. Symbols are the same as in Figure 2.

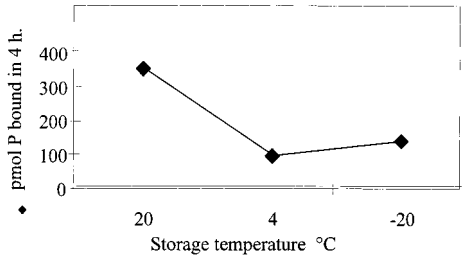

Figure 4. Effect of temperature storage on the phosphate incorporation by phosphocaseinate. Freshly prepared phosphocaseinate solutions (25 mg/mL) has been stored 24 h at the desired temperature (4 or −20 °C). Aliquot fractions have been sampled and phosphorylated using [γ-^{32}P]ATP 4 h by rspCK2α.

Table 4. Comparison of Physical Characteristics of Overphosphorylated Phosphocaseinate vs Native Phosphocaseinate[a]

	control	overphosphorylated sample
proteins solubility (%)	65 ± 11	87 ± 4

[a] Solubility was estimated on sample submitted to 2 h centrifugation at 70000*g* and 20 °C. $N = 4$ experiments.

fact that the commercial proteins are partially dephosphorylated during their purification and that some S, T, or Y may be phosphorylated in noncanonical consensus sequences. (Ralet et al., 1999). Maximal phosphorylation of purified α-caseins by rspCK2α is almost achieved in vitro, as we obtain a total phosphate content of 8.65 P/mol upon the action of rspCK2α, the maximal theoretical incorporation value being 11 to 12 (Table 2). *κ*-Caseins was the poorest substrate (0.29 pmol/mol). Phosphocaseinate phosphate content was increased 1.31 times upon the action of rspCK2α. Phosphate incorporation is higher in isolated caseins than in phosphocaseinate.

Higher incorporations into PPCN could be achieved at pH 8, which is the optimum pH of protein kinase CK2 (Chardot et al., 1995). Under these conditions, phosphocaseinate will lose its micellar structure. The combination of different kinases exhibiting different specificities will be a mean to achieve high level of phosphate incorporation. cAMP-dependent protein kinase could add one more P to α-S_1-casein, but even in that case, not all S, T, or Y of caseins will be phosphorylated. Using rspCK2α, a significant amount of phosphate is incorporated into the isolated caseins; especially in the case of *β*-casein, overphosphorylation with respect to the theoretical expected incorporation is observed. PPCN, supposed to exhibit a native-like structure, is also overphosphorylated with the main target protein being *β*-casein.

To our knowledge, the enzymatic phosphorylation of micellar caseins using enzymatic processes has not been described. Phosphorylation of micellar caseins appears to be sequential with *β*-casein being the first substrate labeled, followed by α-casein and *κ*-casein. The order of affinity of the enzyme for its substrates is the same whether the proteins are included in micelles or not. The enzyme is behaving as if the micelles did not exist or as if their structure was loose enough to let it get in to phosphorylate *β*-caseins, which are considered generally less accessible relative to other caseins. The model proposed by Horne et al. (1989) seems consistent with these experimental data as it does not directly propose the existence of micelles. An attempt to increase the level of phosphate incorporation by increasing the amount of "free" *β*-casein upon storage at 4 °C and by phosphorylating at 30 °C failed. Moreover, upon this treatment, total phosphate incorporation into PPCN diminished. For temperatures higher than 8−9 °C (phosphorylation is performed at 30 °C), *β*-casein began to reenter into the micelle. As phosphorylation yield is decreased, this means that the structure obtained upon rewarming is different from the original one.

The difference in casein reactivity has already been observed by different authors, among them Popineau et al. (1995), who found that the reactivity of purified caseins toward chemical phosphorylating agents was higher than the reactivity of micellar caseins. Individual casein components were dephosphorylated more rapidly than mixtures of α_s- and *β*-caseins (Lorient and Linden, 1976) or micellar casein (Li Chan and Nakai, 1989). This

difference in casein reactivity certainly is due to steric hindrance in the micelle.

The affinity of the micelle for water is, as expected, enhanced after phosphorylation. Solubility increased by 33%. Casein renneting is very sensitive to the conditions of the medium, especially heat treatment and pH have to be checked with great attention, as they have a great importance on the properties of caseins. We always took great care in controlling and eventually adjusting the pH of our solutions, especially as phosphorylation of PPCN was performed in a nonbuffered medium. Upon overphosphorylation, the action of rennet on PPCN was delayed. The action of rennet may be delayed due to the unfolding of β-casein, masking the rennet target site. An increased hydrophilicity of the micelle may also impair the action of chymosin as this enzyme is catalyzing hydrolysis of hydrophobic residues of κ-casein. The addition of phosphate to caseins increases the number of phosphocalcium bridges, stabilizing the proteic structure; this also can explain an increased renetting time. The effect of enzymatic phosphorylation on the sensitivity of micellar caseins to rennet have to our knowledge not been described. Contradictory results have been published upon the effect of casein dephosphorylation on the action of rennet (Pearse, 1986; Van Hekken, 1994). Enzymatic phosphorylation is a mild way to modify caseins. The physical properties and reactivity of micellar caseins are strongly affected upon phosphorylation. Further work will be needed to better investigate and understand the structure of the micelle as a function of its phosphorylation state.

ABBREVIATIONS USED

CK2, type II casein kinase; IPTG, isopropylthio-β-D-galactoside, rspCK2α, recombinant protein kinase CK2 catalytic subunit from *S. pombe*; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis, PMSF, phenylmethanesulfonyl fluoride; PPCN, phosphocaseinate; TCA, trichloracetic acid; TEA, triethanolamine.

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LITERATURE CITED

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