

DEPHOSPHORYLATION OF α_s - AND κ -CASEINS AND ITS EFFECT ON
MICELLE STABILITY IN THE κ - α_s -CASEIN SYSTEM

1937

SUMMARY

α_s - and κ -Caseins, obtained from commercial pooled milk, were dephosphorylated with a phosphoprotein phosphatase prepared from beef spleen. Ultracentrifuge studies at pH 7.0 on 1:4 (κ : α_s) weight ratios of the dephosphorylated proteins gave patterns consisting of a major peak, $S_{20} = 7.5$, and a barely discernible minor peak, $S_{20} = 4.4$. For a similar mixture that had been maintained at pH 12.0 for 10 min before ultracentrifugation at pH 7.0 a single peak, $S_{20} = 7.5$, was obtained. The native κ - and α_s -caseins in 1:4 (κ : α_s) weight ratios gave only a single peak, $S_{20} = 7.5$, under both of the above conditions.

Micelle stability of mixtures of the dephosphorylated proteins with the native κ - and α_s -caseins were compared. The stabilizing power of κ -casein was not changed significantly by dephosphorylation, but the dephosphorylation of α_s -casein impaired drastically its ability to be stabilized by κ -casein in the presence of 0.02 M CaCl_2 at pH 7.0.

From the evidence accumulated by Waugh et al. (8, 11, 12), it is suggested that the α -casein complex, as a micellar unit, is an important factor involved in the stabilization of calcium caseinate micelles in milk. Waugh has proposed a model of the complex (11) where the primary interaction between three molecules of α_s -casein and one molecule of κ -casein is attributed to the formation of hydrogen bonds and the interaction of the abundant non-polar side chains of casein (10). The introduction of calcium ions, in his opinion, forms calcium-esterified phosphate cross-linkages among the α_s -casein molecules of the same complex, thus conferring unusual stability upon the entire structure.

In this paper, we are concerned with the effect of dephosphorylation of κ - and α_s -caseins on their ability to form a stable micelle system. Purified κ - and α_s -casein preparations were dephosphorylated with a phosphoprotein phosphatase derived from beef spleen by a modification of the method of Revel and Racker (5). Conditions were found that afforded almost complete dephosphorylation of the two caseins. The dephosphorylated caseins were examined for their ability to form complexes in the ultracentrifuge and also for their ability to form stable micelles in the presence of calcium ions.

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EXPERIMENTAL PROCEDURES

Electrophoresis. Starch gel electrophoresis at pH 8.6 in 7.0 M urea was performed according to the method of Wake and Baldwin (9). Free boundary electrophoresis was carried out in the Perkin-Elmer² Model 38A apparatus employing the 2-ml Tiselius cell at 2°C in veronal buffer, pH 8.6, $\mu = 0.1$ and in phosphate buffer, pH 7.0, $\mu = 0.1$.

Ultracentrifugation. Sedimentation studies of mixtures of dephosphorylated κ - and α_s -caseins, in a 1 κ :4 α_s weight ratio, were conducted in a Spinco Model E ultracentrifuge² at 25°C. The total protein concentration was 1% in phosphate buffer, pH 7.0, $\mu = 0.2$. Two such mixtures were ultracentrifuged at pH 7.0, but one of them was maintained at pH 12.0 for 10 min before returning to the original pH. The same experiments were performed on mixtures of the native κ - and α_s -caseins.

Stabilization tests. The ability of the dephosphorylated κ - and α_s -caseins to form stable micelles in the presence of 0.02 M calcium chloride at pH 7.0 was determined by the method of Zittle (13).

κ -Casein. κ -Casein was prepared from commercial pooled milk by the method of McKenzie and Wake (4). The κ -casein was essentially free of contaminants in starch gel electrophoresis and sedimented as a single component in

² It is not implied the U. S. Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

the analytical ultracentrifuge, $S_{20} = 14.0$, in phosphate buffer at pH 7.0, $\mu = 0.20$. Lyophilized κ -casein contained 0.14% phosphorus. Unpublished studies in this laboratory suggest that κ -casein may vary in milks from individual cows. This may account for the different phosphorus values reported for κ -casein obtained from different sources (1, 11).

α_s -Casein. α_s -Casein was precipitated twice at 2 C, 0.4 M Ca^{++} , pH 7.0 from whole α -casein prepared from commercial pooled milk by the phosphate buffer, pH 7.0, $\mu = 0.2$. Two such mixture method of Hipp et al. (2). It was found essentially free of contaminants in starch gel electrophoresis, migrating as a single zone, and sedimented as a single asymmetric component in the ultracentrifuge, $S_{20} = 4.4$, in phosphate buffer pH 7.0, $\mu = 0.20$. The phosphorus content of the lyophilized material was 1.0%. Thompson et al. (7) have demonstrated three α_s -casein variants in the milks from individual cows, and that α_s -B/B is the predominant phenotype. The pooled α_s -casein used in this study appears to be principally B/B, but may contain small amounts of A or C, or both.

DEAE cellulose. This product was purchased from Eastman Organic Chemicals.²

Analytical procedures. Organic phosphorus was determined by the method of Sumner (6), after digesting 10-mg and 20-mg samples of the enzyme-reacted α_s - and κ -casein with 1:1 H_2SO_4 and oxidizing with 30% H_2O_2 . Ten per cent TCA soluble nitrogenous material released by the action of phosphoprotein phosphatase on κ - and α_s -caseins was measured by the method of Koch and McMeekin (3), which is sensitive to 0.3 mg NPN. Phosphatase assays were carried out in a system of 1 ml 0.02 M acetate buffer, pH 5.8, 0.6 ml 5% whole casein, pH 7.0, 0.2 ml 0.08 M neutralized ascorbic acid, 0.2 ml of the enzyme solution. After 30 min 2 ml of cold 20% TCA was added and the liberated phosphorus was determined on the supernatant after centrifugation. By this method, the milliliters of enzyme solution necessary to almost completely dephosphorylate a given amount of casein in 1 or 2 hr was determined.

Preparation of the phosphoprotein phosphatase. The method described by Revel and Racker (5) was followed up to the second $(\text{NH}_4)_2\text{SO}_4$ precipitation. At this step, no precipitate was formed and additional $(\text{NH}_4)_2\text{SO}_4$ was added to completely saturate the solution. This treatment did not precipitate the protein, so the saturated solution was continuously dialyzed against 16 liters of distilled water with four changes over three days. The precipitate which formed on dialysis was centrifuged, ex-

tracted with 0.5 M NaCl—0.2 M sodium acetate solution, pH 5.0, and centrifuged again at 4,000 rpm. The resulting supernatant was then carried through the protamine sulfate precipitation and the remainder of the procedure as described by Revel and Racker.

Dephosphorylation. Dephosphorylation of κ - and α_s -casein was carried out in a system similar to that described above for the phosphatase assay, but with the volumes of the different reagents increased proportionally to accommodate 500 mg of casein in a 2.5% solution. The reaction was stopped by acidifying to pH 4.7 and centrifuging. The product was reprecipitated, washed five times with distilled water, redissolved at pH 8.0, dialyzed and lyophilized. Five-hundred-milligram portions of α_s -casein were enzymatically dephosphorylated for 60-min and 75-min reaction periods. Five hundred milligrams of κ -casein was dephosphorylated in a 60-min reaction period. The 10% TCA soluble nitrogenous material was estimated after a 2-hr reaction period in a system containing 500 mg of α_s -casein. A similar system containing no enzyme was the control.

RESULTS AND DISCUSSION

Maximum precipitation of the dephosphorylated α_s -casein preparations occurred at pH's varying from 5.0 to 5.2. These pH values are higher than the usual value of pH 4.7. The dephosphorylated κ -casein did not exhibit this effect. This may be ascribed to the much lower electronegative phosphate content of native κ -casein compared to native α_s -casein.

Starch gel electrophoresis gave a series of bands for a 91% dephosphorylated α_s -casein (60-min reaction). The 96% dephosphorylated α_s -casein (75-min reaction) exhibited much less banding and with a considerable reduction in mobility compared to the single band obtained for the native α_s -casein employed in this experiment. The 100% dephosphorylated κ -casein (60-min reaction) gave a single characteristically diffuse band on starch gel electrophoresis, slightly reduced in mobility compared to the native κ -casein. The free boundary electrophoretic patterns of 96% dephosphorylated α_s -casein and 100% dephosphorylated κ -casein appeared as single, slightly skewed, peaks.

The fact that the banding, observed in starch gel patterns of the dephosphorylated proteins, decreases as the protein is increasingly dephosphorylated, and that no NPN is released by the action of phosphoprotein phosphatase on

α_s -casein in 2 hr, is evidence that proteolysis has not occurred.³

The ultracentrifuge patterns at pH 7.0 disclosed that dephosphorylated κ - and α_s -caseins in a weight ratio of 1 κ :4 α_s formed a complex ($S_{20} = 7.5$). However, in the pattern of the mixture maintained at pH 7.0, a barely discernible minor component sedimented with a velocity characteristic of α_s -casein ($S_{20} = 4.4$). The mixture subjected to pH 12.0 for 10 min and returned to pH 7.0 appeared as a single skewed peak ($S_{20} = 7.5$). A peak corresponding to uncomplexed κ -casein ($S_{20} = 14.0$) was not observed in either run.

The ultracentrifuge pattern of the native κ - and α_s -caseins in the same weight ratio at pH 7.0 appeared as a single peak ($S_{20} = 7.5$), both before and after titration to pH 12.0. The minor peak ($S_{20} = 4.4$) in the pattern of the dephosphorylated casein mixture maintained at pH 7.0 corresponds to uncomplexed α_s -casein. This presence of a minute quantity of uncomplexed dephosphorylated α_s -casein is evidence that the capacity to form a stable complex is only slightly altered by dephosphorylation.

The stability tests (Figure 1) compare native κ -casein with dephosphorylated κ -casein in ability to stabilize native α_s -casein, 91 and 96% dephosphorylated α_s -casein. It is evident from the figure that the stabilizing ability of κ -casein is not affected significantly by dephosphorylation, but that dephosphorylation of α_s -casein drastically impairs its ability to be stabilized by κ -casein in 0.02 M CaCl_2 at pH 7.0.

It is concluded that the formation of stoichiometric κ - α_s -casein complexes is not influenced noticeably by the presence of esterified phosphate groups, but probably depends upon the interaction of nonpolar side chains and the formation of strong hydrogen bonds as suggested by Waugh (10). Furthermore, the interaction of calcium ions and esterified phosphate groups between κ - and α_s -casein molecules of the complex could not be an important factor leading to the growth of stable micelles. It is inferred, however, that the growth of stable micelles from the κ - α_s -casein complexes in the presence of calcium ions is dependent upon the interaction of calcium ions and esterified phosphate groups among the α_s -casein mole-

³ When dephosphorylation of α_s -casein was performed in the presence of diisopropyl-fluoro-phosphate (DFP), to retard proteolytic activity, multiple banding of α_s -casein persisted when examined on starch-gel. This fact is added evidence that the banding observed in the starch-gel patterns of the α_s -casein reacted with non-DFP treated enzyme is not a consequence of proteolysis.

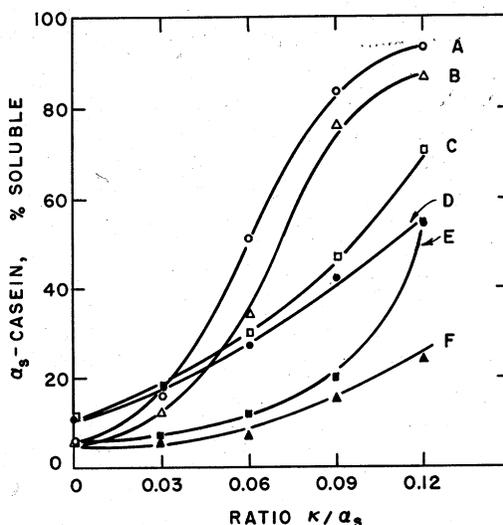


Fig. 1. Stabilization of α_s - and dephosphorylated α_s -casein by κ - and dephosphorylated κ -casein in 0.02 M CaCl_2 at pH 7.0.

- A— κ -casein + α_s -casein.
 B—100% dephosphorylated κ -casein + α_s -casein.
 C—100% dephosphorylated κ -casein + 91% dephosphorylated α_s -casein.
 D— κ -casein + 91% dephosphorylated α_s -casein.
 E— κ -casein + 96% dephosphorylated α_s -casein.
 F—100% dephosphorylated κ -casein + 96% dephosphorylated α_s -casein.

cules of the same complex. Whether this interaction leads to the establishment of calcium-esterified phosphate cross linkages as proposed by Waugh (11) remains to be clarified.

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