AMINO TERMINAL SEQUENCES OF THE PRECURSORS OF OVINE CASEINS

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SUMMARY

The amino terminal sequences of the 4 caseins synthesized by translation of ovine mammary mRNAs in a wheat germ cell-free system have been investigated by automated Edman degradation. The 3 "calcium-sensitive" caseins (α_{s1}, α_{s2} and β) and κ-casein were synthesized as precaseins with amino terminal hydrophobic extensions of 15 and 21 amino acid residues respectively, resembling "signal peptides" of other secretory proteins. The extra pieces of the 4 caseins, which start with a methionyl residue, end with an alanyl residue which may be one of the signals recognized by the mammary membrane-bound enzyme responsible for the specific cleavage of precaseins. The amino terminal extensions of α_{s1}, α_{s2} and β-caseins show a high degree of homology suggesting that they have derived from a common ancestor.

INTRODUCTION

Caseins are the major secretory proteins synthesized by the lactating mammary gland. In ruminants they account for up to 80 percent of the total lactoprotein content. They are stored and excreted as stable aggregates called micelles containing up to several ten thousand casein molecules. Micelles of bovine milk which have been extensively investigated, are essentially intricate assemblies of 4 phosphoproteins, α_{s1}-, α_{s2}-, β- and κ-caseins, in the proportion of approximatively 38, 11, 38 and 13 percent respectively (1). The first 3 caseins which are "calcium-sensitive" are stabilized when they copolymerize with κ-casein hence its name of micelle stabilizer (2). The primary structures of the 4 bovine caseins, which are coded for by 4 structural genes located close together in a cluster (3, 4), are now well established (5-8). Four species of caseins, similar to their bovine counterparts, have been isolated from ovine milk (9) and the amino acid sequences of ovine κ- and β-caseins have been elucidated (10, 11).

Previous studies at this Institute have shown that milk proteins are essentially synthesized on membrane-bound polysomes (12, 13) and that the translation of mammary mRNAs isolated from such polysomes leads to the synthesis of different species of caseins in a reticulocyte lysate (14, 15). The *in vitro* synthesis of caseins from different origins (rabbit, rat, mouse, guinea pig) using various cell-free systems have also been reported (16-18).

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Whenever the translation products were examined in SDS gel, no difference in size was observed between the *in vitro* products and the corresponding authentic caseins.

In this paper, we demonstrate that the initial translation products of ovine mammary mRNAs coding for the 3 "calcium-sensitive" caseins and κ-casein are larger molecules containing hydrophobic amino terminal extensions of 15 and 21 amino acid residues respectively, similar to the "signal peptides" described for various secretory proteins (19 - 26) as well as some membrane-bound proteins (27).

MATERIALS AND METHODS

Preparation of a wheat germ extract. The wheat germ extract was prepared according to Davies and Kaesberg (28) using the procedure described by Benicourt and Haenni (29) modified as follows : the 17,500 rpm supernatant was filtered on a Sephadex G-25 column (1.5 x 30 cm) equilibrated with a solution containing 10 mM Hepes pH 7.5, 90 mM K acetate, 1 mM Mg acetate and 1 mM DTE. The peak of the turbid fraction was stored at -80°C as small aliquots.

Preparation of antibodies. Antibodies against bovine β-casein, ovine κ- and α_s-caseins were prepared as previously described (15). α_s-casein was a mixture of both α_{s1}- and α_{s2}-caseins in the proportion of approximatively 80 and 20 percent respectively.

Protein synthesis by a wheat germ lysate. Poly A RNAs isolated from bound polysomes of a lactating ewe mammary gland (14) were translated at the concentration of 0.01 to 0.02 A_{260} in a reaction mixture containing per 100 µl, 25 µl S23 wheat germ lysate, 20 mM Hepes pH 7.5, 100 mM K acetate, 2 mM Mg acetate, 300 µM spermidine HCl, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 5 µg of creatine kinase, 20 mM of unlabeled amino acids. The radioactive amino acids (either {^{3}H} or {^{14}C} amino acids purchased from CEA France) were added at the concentration of 5 µM except for methionine (obtained from Radiochemical Centre Amersham), added at 1 µM. Reaction vessels were incubated at 26°C for 2 hours. For measuring the amino acid incorporation in the total proteins 1 ml of 5% CCl_3COOH containing 0.2% of the cold amino acids was added to 5 µl aliquots which were subsequently heated at 90°C for 15 mn. The precipitates were collected on Whatman GF/C glass filters. The radioactivity was counted in toluene-based scintillation fluid using a Packard model 3375 liquid scintillator.

Immunoprecipitation. The incubated reaction mixture was centrifuged at 105,000 g for 1 hour. A stock solution of phosphate buffered saline (PBS), containing 100 mM Na phosphate pH 7.2, NaCl 1.4 M, 100 mM of the appropriate amino acids, 10% DOC and 10% Triton X100, was added to the resulting supernatant to obtain a final concentration of 10 mM Na phosphate, 0.14 M NaCl, 10 mM amino acids, 1% DOC and 1% Triton X100. This was followed by the addition of 5 µg of the appropriate casein carrier and 100 µl of the appropriate specific immunserum. Then, the mixture was incubated at 37°C for 1 hour. The immunoprecipitate was collected by centrifugation at 15,000 rpm for 5 mn using a Beckman model 152 microfuge, through a 800 µl cushion of 1M sucrose in PBS containing 1% Triton X100 and 1% DOC. The pellet was resuspended in PBS and recentrifuged as described above. The procedure was repeated once and the final precipitate lyophilised.

Gel electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed on gel slab gradients (10 to 20%) using the buffer system described by Laemmli (30). The gels were subjected to either an auto-

radiography or a fluorography according to Bonner and Laskey (31).

Amino acid sequence determination. The lyophilized immunoprecipitate containing the translation product radiolabeled with one or two amino acids was dissolved in 300 µl trifluoroacetic acid. After removal of an aliquot for determination of total radioactivity, the sample supplemented with 6 mg of water buffalo β-lactoglobulin carrier was applied to a Beckman updated model 890 B sequencer. The automated Edman degradation was performed using the Beckman "dimethylbenzylamine" program 102473, each run being preceded by a blank cycle (without phenyl isothiocyanate) used to improve the quality of the film and discard any radioactive contaminant. The thiazolinone derivatives recovered at each sequencer cycle were converted to phenylthiohydantoin amino acids in N.HCl, extracted with ethyl acetate and dried down under nitrogen. The extract was redissolved in an appropriate volume of ethyl acetate. An aliquot was removed and analyzed by gas chromatography to monitor the efficiency of the carrier degradation and determine the repetitive yield. Another aliquot of the extract was counted in toluene-based scintillation fluid. The remaining portion was used for the identification of the labeled PTH amino acids which were cochromatographied with appropriate PTH amino acid carriers on silica gel. The spots were scraped off the plates and counted in the liquid scintillator. The aqueous phase was counted whenever a sample contained radiolabeled arginine.

RESULTS

Translation of polyadenylated RNAs, isolated from bound polysomes of ewe mammary gland, by a wheat germ cell-free system causes an important stimulation of polypeptide synthesis (5 to 30 fold depending upon labeled amino-acids). Analysis of the translation products by SDS polyacrylamide gel electrophoresis and subsequent autoradiography yielded a major band with an apparent molecular weight of 26000-27000 daltons. Immunoprecipitates obtained with β- and α_s-caseins antibodies were undistinguisable from the major band and showed an electrophoretic migration very close to those of the authentic caseins (Fig. 1). As the mature caseins are phosphorylated in contrast with their immature counterparts, such a slight difference in electrophoretic mobility does not suggest by itself the presence of precursors since the occurence of anionic charges in a protein can affect considerably its rate of migration in SDS gel (32).

To demonstrate the presence of casein precursors, amino acid sequence analyses were carried out on each *in vitro* synthesized casein radiolabeled with one or two different amino acids each time. Fig. 2A shows the amount of radioactivity recovered at each cycle of the Edman degradations of 4 immunoprecipitates of β-casein radiolabeled with {^{35}S}-methionine, {^{3}H}-leucine, {^{3}H}-glutamic acid and {^{3}H}-valine respectively. The results demonstrate clearly the occurrence of a β-casein precursor since leucyl residues occur at positions 4, 6, 9, 12, 14 and 21 whereas the amino terminal sequence of authentic β-casein contains a single leucyl residue at position 6 (11) (Fig. 3). Furthermore, it is obvious that the amino terminal extension is 15 residues long since the positions of glutamyl residues in the precursor, 17, 19, 20, 26

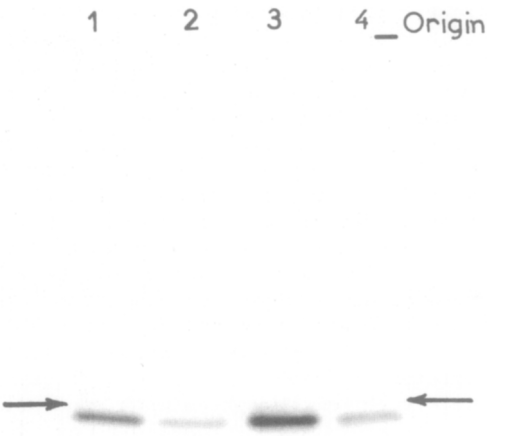

FIG. 1. Analysis, by sodium dodecyl sulfate gel electrophoresis and subsequent autoradiography, of mammary Poly(A) RNAs translation products and their immunoprecipitates : 1 and 2. Ovine immunoprecipitates obtained with α_s- and β-caseins antibodies respectively. 3. Translation products of ovine mammary Poly(A) RNAs. 4. Immunoprecipitates of bovine β-casein translated by a wheat germ cell-free system programmed by bovine mammary Poly(A) RNAs.
Positions of authentic α_s- and β-caseins are indicated by arrows.

and 29, match perfectly with those occupied by this residue in authentic ovine β-casein, 2, 4, 5, 11 and 14 respectively. Similar data obtained with other amino acids have allowed us to establish the sequence of all but one residues of the signal peptide of β-casein (Fig. 3).

A similar approach was used to demonstrate the occurrence of precursors for the other caseins. However, in the case of α_{s1}- and α_{s2}-caseins, the primary structure analysis was carried out simultaneously on immunoprecipitates of both translated products which were in the average ratio 4:1. Furthermore, it was assumed that amino terminal sequences of authentic ovine α_{s1}-and

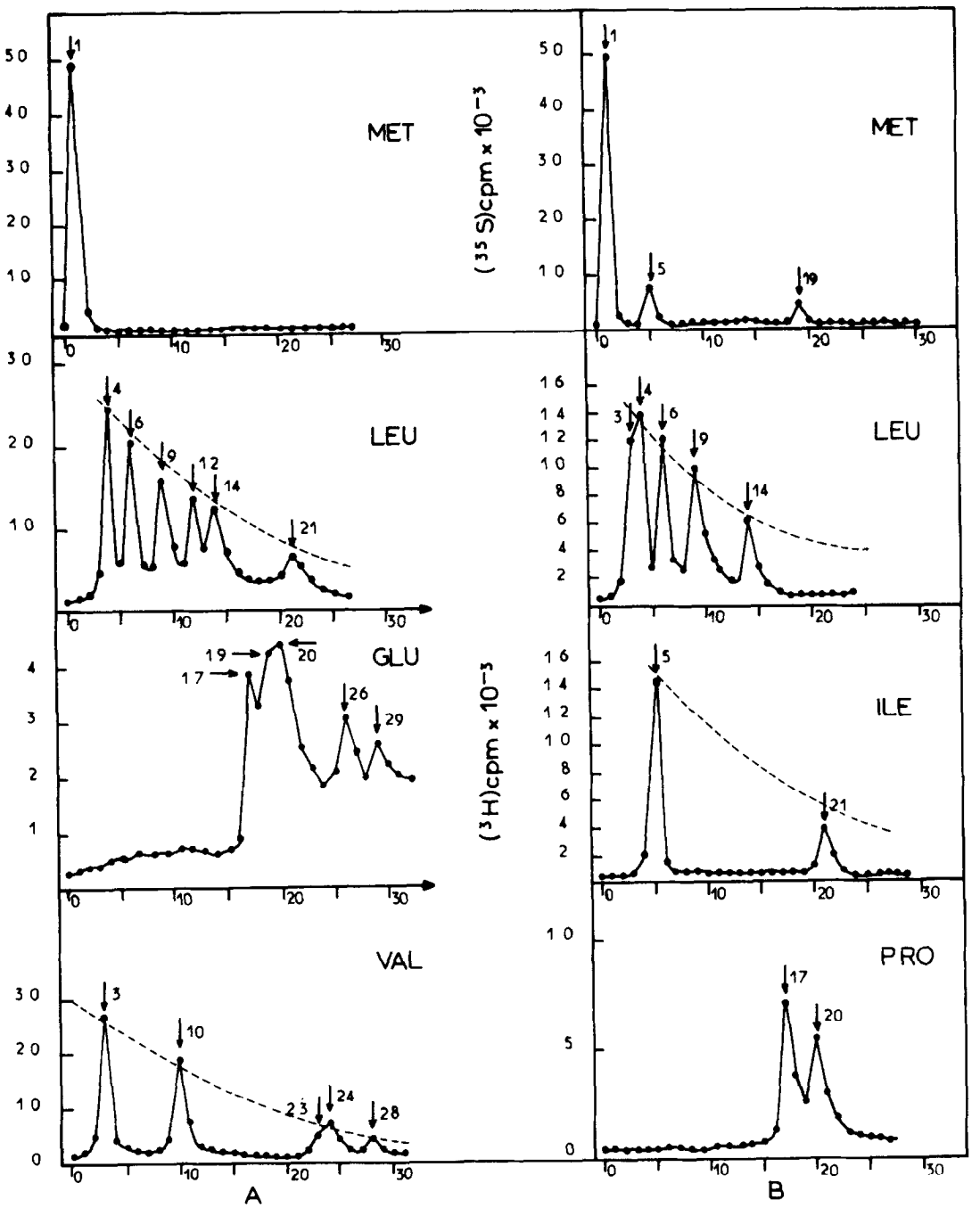

FIG. 2. Typical sequential Edman degradations of immunoprecipitates of *in vitro* translated β-casein (A) and α_{s1}- + α_{s2}-caseins (B) radiolabeled with one amino acid each time. The dashed curve lines indicate the theoretical yields of radioactivity calculated from the repetitive yields (94%) of the degradation of the carrier water buffalo β-lactoglobulin and normalized to the first radioactive peak. Sequence positions assigned to various amino acids are indicated by arrows. Cycle zero represents the blank cycle as described in *Materials and Methods*.

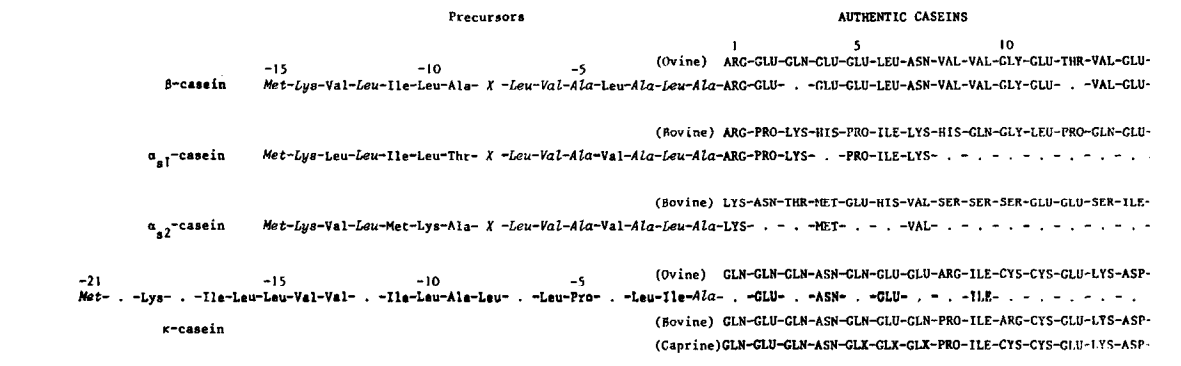

FIG. 3. Partial amino terminal sequences of precursors of the 4 ovine caseins α_{s1}, α_{s2}, β and κ, deduced from radiosequence data.
The amino acid sequence of pre-α_{s2}-casein should be considered as a tentative one (*see results*). A glutamyl residue, instead of a glutaminyl residue as expected from Jollès sequence data (10), has been identified at position 23 of the polypeptide chain of ovine pre-κ-casein. Residues in *italics* are common to the 3 caseins α_{s1}, α_{s2}, and β. Residues in CAPITALS are those of authentic caseins. Unidentified residues are marked by a dot.
Sequence analyses were performed on precursors of β and α_{s1} + α_{s2}-caseins radiolabeled each time with one or two of the following residues : (^{14}C)Thr, (^{14}C)Phe, (^{3}H)Ser, (^{3}H)Glu, (^{3}H)Pro, (^{3}H)Gly, (^{3}H)Ala, (^{3}H)Val, (^{35}S)Met, (^{3}H)Ile, (^{3}H)Leu, (^{3}H)Asn, (^{3}H)Tyr, (^{3}H)Lys, (^{3}H)Trp, and (^{3}H)Arg. Thus, *X* is one of the remaining amino acids His, Asp, Gln and Cys. In the case of κ-casein, only a few samples radiolabeled with (^{3}H)Asn, (^{3}H)Glu, (^{3}H)Pro, (^{3}H)Ala, (^{3}H)Val, (^{3}H)Ile, (^{35}S)Met, (^{3}H)Leu and (^{3}H)Lys respectively have been analyzed.

α_{s2}-caseins were similar to those of their bovine counterparts. Fig. 2B shows some results obtained for these 2 caseins. The occurrence of isoleucyl and prolyl residues at cycles 21 and 17,20 respectively (positions which match perfectly with those occupied by these residues in authentic α_{s1}-casein) together with the high radioactivity yield clearly indicate that a 15 residues long extra piece precedes the N-terminal residue of authentic α_{s1}-casein. This was confirmed by subsequent analyses of precursors radiolabeled with other amino acids (Fig. 3). The partial amino acid sequence of pre-α_{s2}-casein was determined by looking at the small peaks of radioactivity. For example, the heights of the methionine peaks 5 and 19 are consistent with the presence of methionyl residues at positions 5 and 19 in the minor component α_{s2} and it was assumed that methionine 19 was that at position 4 in the authentic α_{s2}-casein. The subsequent identification of small peaks of lysine and valine at cycles 6, 16 and 3,22 respectively, was in perfect accordance with the presence of a precursor of α_{s2}-casein with an amino terminal extension of 15 residues as shown Fig. 3. Nevertheless, the partial amino acid sequence of

TABLE 1

EFFECTS OF SODIUM NITROPRUSSIDE (SNP) AND ACETYLCHOLINE (ACh) ON CYCLIC NUCLEOTIDE LEVELS AND CONTRACTILE FORCE IN CAT ATRIUM

Treatment	N	Cyclic GMP (pmoles/g tissue)	Cyclic AMP (pmoles/g tissue)	Contractile Force (% change)
Control	7	12.7 ± 2.3	382 ± 88	
SNP (10^{-4}M, 60 sec)	7	218.3 ± 30.9*	383 ± 77	+11.4 ± 1.5*
Control	8	6.8 ± 1.1	750 ± 80	
ACh ($5x10^{-8}$M, 15 sec)	8	6.8 ± 1.5	607 ±103	-14.9 ± 3.3*
Control	6	9.5 ± 1.4	484 ± 48	
ACh ($5x10^{-8}$M, 60 sec)	6	8.3 ± 1.5	394 ± 59	-19.5 ± 7.7*
Control	8	7.6 ± 0.9	471 ± 116	
ACh ($5x10^{-7}$M, 15 sec)	8	26.9 ± 8.4*	463 ± 94	-41.8 ± 3.3*
Control	8	14.5 ± 2.9	343 ± 44	
ACh ($5x10^{-7}$M, 60 sec)	8	13.3 ± 2.3	332 ± 38	-63.0 ± 6.5*

*Significantly different from the corresponding controls ($p < 0.05$ or better). Results are expressed as means ± S.E. of the number of samples indicated (N). Percent change in contractile force represents the difference between pre-drug twitch tension and twitch tension at the time the preparations were frozen.

pre-exposure of the muscles for 1 min to 10^{-4} M sodium nitroprusside, the same concentration of acetylcholine decreased contractile force by 30.3 ± 6.3% (N = 8). Thus, the pretreatment of the atrial strips with sodium nitroprusside had no effect on the negative inotropic response of the muscles to acetylcholine.

DISCUSSION

As mentioned earlier, if an increase in cyclic GMP levels is actually responsible for the negative inotropic effect of acetylcholine in atrial myocardium, then any agent which can directly increase atrial cyclic GMP levels should also have a negative inotropic action. The results of the present study indicate that this is not the case. Marked increases in cyclic GMP levels could be produced in cat atrial strips by concentrations of sodium nitroprusside that caused a slight increase, rather than a decrease, in con-

the lumen of the rough endoplasmic reticulum to prevent any precipitation of other caseins as they are released from the membrane. In this respect, it is attractive to speculate that the differences observed between the amino terminal extensions of κ-casein and the other caseins may be related to a timing or (and) a compartimentalization in the transfer of these proteins across the microsomal membrane and the subsequent removal of the signal peptide. Preliminary experiments have shown that precaseins are converted into authentic caseins when casein mRNAs are translated in the presence of mammary microsomal membranes, but it seems essential to determine whether or not pre-κ-casein is cleaved in the same manner as the other caseins. In this respect, the knowledge of the amino terminal sequences of other *in vitro* synthesized secretory lactoproteins such as β-lactoglobulin and α-lactalbumin will give valuable information.

On the other hand, some of the signal peptides which are released into the lumen of the rough endoplasmic reticulum might occur in milk and their possible occurrence is under investigation.

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