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AMINO TERMINAL SEQUENCE OF THE PRECURSOR OF OVINE α-LACTALBUMIN

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SUMMARY

Ovine mammary gland mRNAs were translated in a wheat-germ cell-free system in the presence of radioactive amino acids. Automated Edman degradation performed on α-lactalbumin isolated by immunoprecipitation from the mixture of radiolabelled lactoproteins showed the occurrence of an hydrophobic 19 residues long amino terminal extension. The pre-protein represents the primary translation product since the amino terminal methionyl residue was found to be donated by initiator Met-tRNA$_i^{Met}$. Comparison of the signals of ovine α-lactalbumin and hen's egg white lysozyme, two homologous proteins which are thought to be derived from a common ancestor, suggests that the signal region has evolved at least as rapidly as the remaining part of the polypeptide chain.

INTRODUCTION

α-lactalbumin is a secretory protein occurring in lactose-containing milks (1,2). This major whey protein is made up of a single polypeptide chain whose amino acid sequence, determined in bovine, human and guinea pig species (3-5), is similar to that of lysozyme (1) thus suggesting that both proteins have derived from a common ancestor (1). α-lactalbumin, which is glycosylated in some species (6-10), plays an important biological role in controlling lactose synthesis during pregnancy and lactation. This protein was identified in 1967 (11) as the B subunit of lactose synthetase, the enzyme catalyzing the last stage of lactose synthesis:

$$\text{UDP-Galactose} + \text{Glucose} \xrightarrow{Mn^{++}} \text{Lactose} + \text{UDP}$$

α-lactalbumin is a "modifier or specifier protein" able to modify through putative conformational change the substrate specificity of the A subunit located in the Golgi region, thus allowing this galactosyl transferase normally involved in the biosynthesis of glycoproteins to preferentially use glucose instead of N-acetylglucosamine residues as a galactose acceptor (12).

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Early *in vitro* studies have shown that specific secretory milk proteins including α-lactalbumin (13-15) are essentially synthesized on membrane-bound polysomes and that mRNAs isolated from such polysomes faithfully directed the synthesis of the different species of secretory lactoproteins in various cell-free systems [quoted in (16)]. However, examination by SDS gel electrophoresis of the *in vitro* translated products gave no convincing evidence that secretory lactoproteins other than α-lactalbumin (17) were synthesized as larger molecules as predicted from the "signal hypothesis". According to that concept (18,19), proteins destined for exportation are manufactured on polysomes as precursors called pre-proteins with peculiar transient amino terminal extensions responsible for the attachment of functioning ribosomes to the endoplasmic reticulum membranes thus providing the topological conditions for the vectorial transfer of nascent chains into the cisternal space. Amino terminal sequence analyses of many *in vitro* synthesized secretory proteins [(20,21) and quoted in (16)] have provided compelling support for the "signal hypothesis" but it must be pointed out that alternative mechanisms might also be involved in protein secretion as suggested by the lack of any precursor of ovalbumin (22).

Recently, we have undertaken to examine the amino terminal sequences of the six major ovine secretory lactoproteins synthesized by translation of mammary gland mRNAs in a wheat germ cell-free system and subsequently separated from each other by immunoprecipitation. The radiosequence data clearly demonstrated the occurrence of precursors and we have already reported the complete primary structures of the hydrophobic "signals" of the four caseins α_{s1}, α_{s2}, β and κ and of β-lactoglobulin (16,23).

The results reported here demonstrate the occurrence of a precursor of α-lactalbumin, as suggested by Craig *et al* (17) on the basis of the electrophoretic and chromatographic behaviour of *in vitro* synthesized guinea pig α-lactalbumin and its cyanogen bromide fragments, and provide the sequence of the 19 residues long hydrophobic amino terminal extension. In addition we have demonstrated that *in vitro* synthesized α-lactalbumin represents the primary translation product from α-lactalbumin mRNA since the first methionyl residue was found to be exclusively donated by the initiator Met-$tRNA_i^{Met}$.

MATERIALS AND METHODS

Preparation of a wheat germ extract. The wheat germ extract was prepared according to Davies and Kaesberg (24) using the procedure described by Benicourt and Haenni (25) slightly modified (23).

Preparation of antibodies. Antibodies against bovine α-lactalbumin were prepared as previously described (26).

Protein synthesis by a wheat germ lysate and immunoprecipitation of α-lactalbumin. The translation of poly A RNAs isolated from bound polysomes of a lac-

tating ewe mammary gland (27) as well as the immunoprecipitation of α-lactalbumin were carried out according to the procedures described in detail elsewhere (23).

Gel electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed on gel slab gradients (10 to 20 percent) using the buffer system described by Laemmli (28). The gels were subjected to either an autoradiography or a fluorography according to Bonner and Laskey (29).

Preparation of initiator Met-tRNA$_i^{Met}$ and elongator Met-tRNA$_m^{Met}$. These tRNAs were purified using the methods early described (30) and acylated with [^{35}S]Met in the presence of pure E.Coli methionyl t-RNA synthetase and partially purified methionyl t-RNA synthetase from rat liver respectively.

Amino acid sequence determination. The lyophilized immunoprecipitates containing the *in vitro* translated α-lactalbumin radiolabelled with one amino acid at a time were analysed either separately or in pool in the Beckman updated model 890 B sequencer using the Beckman "dimethylbenzylamine" program 102473. Each sample was supplemented with 5 mg of bovine β-lactoglobulin used as a carrier. The material released at each cycle of the Edman degradation was converted into phenylthiohydantoin amino acids (PTH) which were analysed as follows: An aliquot (1/25) was gas chromatographed to monitor the efficiency of the carrier degradation and calculate the repetitive yield. Another aliquot (1/4 to 1/2) was assayed for radioactivity and counted in toluene-based scintillation fluid in a Packard model 3375 liquid scintillator. The remaining portion was used for the identification of the labelled PTH amino acids which were chromatographed with appropriate PTH amino acid carriers on silica gel (Merck F 254). The spots were scrapped off the plates and counted in the liquid scintillator. The aqueous phase obtained at each conversion cycle was counted whenever a sample contained radiolabelled arginine or histidine. [^{35}S]Cys-labelled α-lactalbumin was reduced and alkylated with iodoacetic acid according to the procedure described by Crestfield *et al* (31) prior to sequence analysis.

Sources of materials. [^{35}S]Met(600) and sixteen [^{3}H]-labelled amino acids were obtained from CEA France: Asp(10), Asn(22), Ser(40), Glu(21), Pro(22), Gly(10), Ala(50), Val(35), Ile(10), Leu(30), Tyr(46), Phe(29), Lys(33), His(46), Trp(4) and Arg(36). [^{3}H]Thr(21) and [^{35}S]Cystine(38) were obtained from New England Nuclear. [^{3}H]Gln(21) was purchased from Radiochemical Centre Amersham. [^{35}S] Cystine was reduced to [^{35}S]Cysteine using the procedure described by Caskey *et al* (32).
Numbers in parentheses refer to specific activity expressed in Ci/mmol.

RESULTS AND DISCUSSION

The occurrence of a presumed pre-α-lactalbumin, as suggested by the slower mobility of *in vitro* synthesized α-lactalbumin co-electrophoresed with its authentic counterpart on SDS polyacrylamide gel (Fig. 1), was demonstrated unambiguously by sequencing separate or pooled immunoprecipitates of *in vitro* translated α-lactalbumin radiolabelled with one amino acid each time. Eleven sequencer runs carried out on a series of 20 samples, each labelled with a different amino acid, enabled us to identify 33 out of the 35 first amino acid residues of the polypeptide chain. Fig. 2 shows representative sequence data from which the amino terminal sequence of pre-α-lactalbumin was established (Fig. 4). The diagrams shown in Fig. 2 require only a few comments: the recovery of radioactivity from silica gels was rather good for PTH derivatives of hydrophobic amino acids (50 to 75%) but less

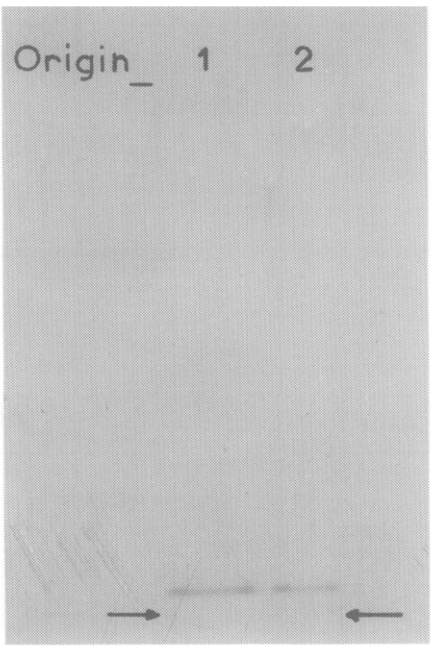


Fig. 1. *Fluorography of an electrophoregram of* [^{3}H]*Leu radiolabelled* α*-lactalbumin immunoprecipitated from the* in vitro *translation products of ovine mammary gland mRNAs.*
Electrophoresis was performed in 10 to 20% polyacrylamide gels in sodium dodecyl sulfate. Lanes 1 and 2 correspond to different concentration of the sample. Arrows indicate the position of authentic α-lactalbumin.

satisfactory for PTH derivatives of hydrophilic amino acids (below 10% in some cases). Nevertheless, discrete and significant peaks of radioactivity were also obtained in the latter case as shown in panels ASP, LYS, GLU and GLN which were readily interpretable. In the GLU panel, the radioactive peaks found at positions 18, 21 and 29 resulted very likely from the deamidation of the glutamine derivatives released at cycles 18, 21 and 29. In contrast, the tryptophan data was not conclusive enough to assign a tryptophanyl residue at position 15 because the level of radioactivity detected at that cycle was too far below that expected from the repetitive yield.

Comparison of the amino terminal sequence deduced from the above data with that of authentic bovine α-lactalbumin (3) provided unequivocal evidence that this protein is initially synthesized as a precursor with a 19 residues long amino terminal extension, assuming that the amino terminal sequence of ovine α-lactalbumin is similar to that of its bovine counterpart (Fig. 4): after position 19, the sequence of amino acid residues is clearly identical with the amino terminal one of authentic α-lactalbumin.

It is noteworthy that pre-α-lactalbumin begins with Met-Met. To ascertain that Met1 is the initiator residue, mammary gland mRNAs were translated in the pre-

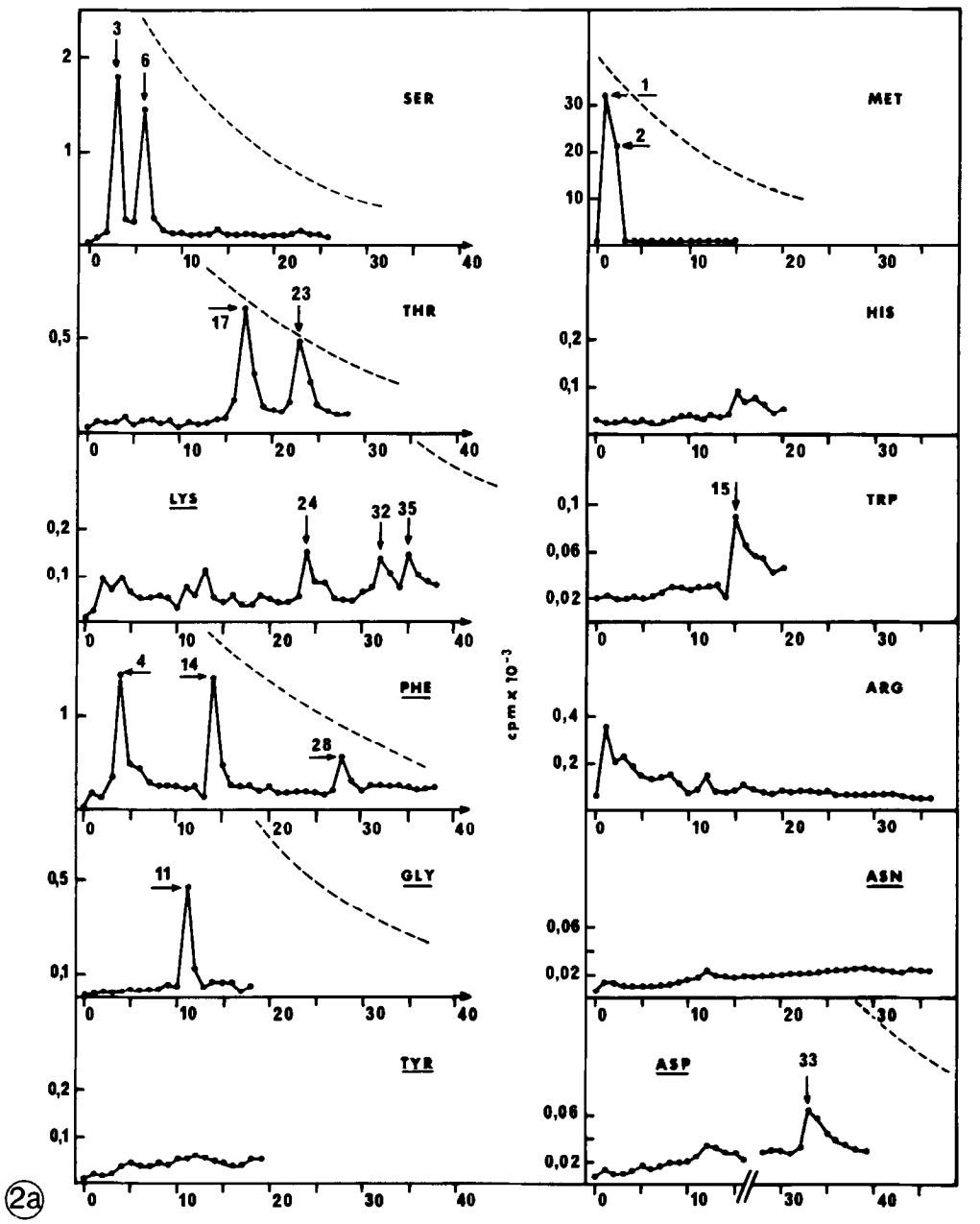
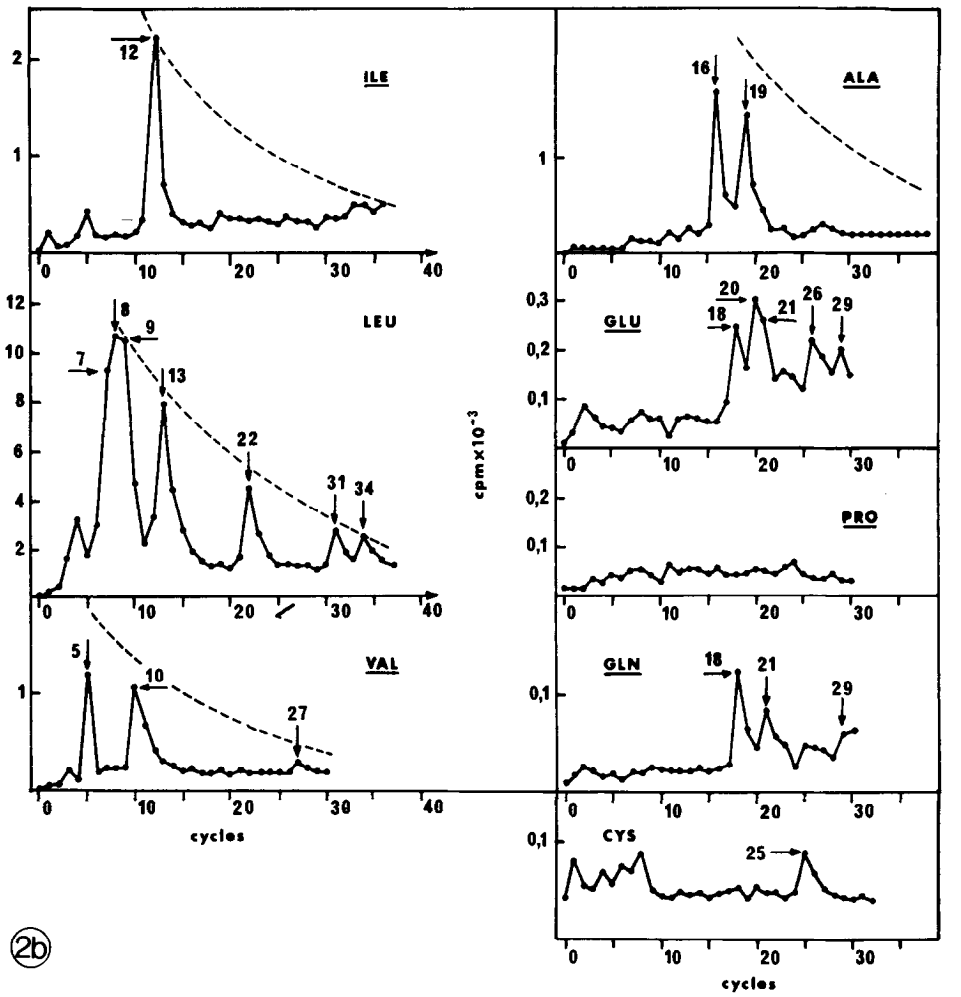


Fig. 2. *Sequence analyses of immunoprecipitates of* in vitro *translated α-lactalbumin radiolabelled with one amino acid each time.*

The data have been corrected neither for background nor any "out of cycle" radioactivity. The dotted curved lines indicate the theoretical yields of radioactivity calculated from the repetitive yields (94 to 96%) of the degradation of the carrier. Cycle zero represents the blank cycle (23). The automated Edman degradation was performed either on separate ([^{35}S]Met; [^{35}S]Cys; [^{3}H]Leu; [^{3}H]Thr; [^{3}H]Ser) or pooled immunoprecipitates ([^{3}H]Pro + [^{3}H]Val + [^{35}S]Met; [^{3}H]His + [^{3}H]Trp; [^{3}H]Ala + [^{3}H]Lys + [^{3}H]Phe;



(2b)

[^{3}H]Gly + [^{3}H]Tyr + [^{3}H]Ile; [^{3}H]Ala + [^{3}H]Glu + [^{3}H]Gln; [^{3}H]Asp + [^{3}H]Asn + [^{3}H]Arg + [^{3}H]Ile).
Amino acids have been underlined whenever a diagram refers to radioactivity recovered from silica gel chromatograms.
Sequence positions assigned to various amino acids are indicated by arrows.

sence of [^{35}S]Met-tRNA$_i^{Met}$ and [^{35}S]Met-tRNA$_m^{Met}$ respectively. Pre-α-lactalbumin was isolated by immunoprecipitation and both samples were then sequenced individually. Fig. 3 shows clearly that Met1 and Met2 were donated by the initiator Met-tRNA$_i^{Met}$ and the elongator Met-tRNA$_m^{Met}$ respectively, thus demonstrating that pre-α-lactalbumin is the primary translation product from α-lactalbumin mRNA.

According to the mechanism of protein secretion outlined in the "signal" concept, the transient amino terminal extension is a kind of "visa" allowing its

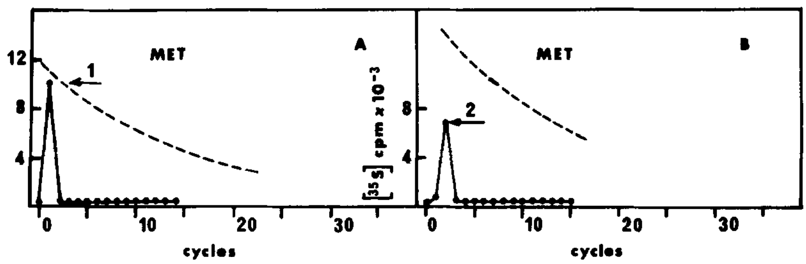


Fig. 3. *Automated Edman degradation of pre-α-lactalbumin labelled with* [^{35}S]*Met donated by initiator* [^{35}S]*Met-tRNA$_i$^{Met} (PANEL A) and elongator* [^{35}S]*Met-tRNA$_m$^{Met} (PANEL B).*

The number over the arrow indicates the position of the methionyl residue in the polypeptide chain of pre-α-lactalbumin.

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                                                        1             5                  10                 15
                            Human α-lactalbumin (4)    LYS-GLN-PHE-THR-LYS-CYS-GLU-LEU-SER-GLN-LEU-LEU-LYS-ASP-ILE-ASP-

                                                                                   TRP
                            Bovine α-lactalbumin (3)   GLU-GLN-LEU-THR-LYS-CYS-GLU-VAL-PHE-ARG-GLU-LEU-LYS-ASP-LEU-LYS-
                                                                                   GLN
1 Ovine pre-α-lactalbumin        10            15          19 20                25                30             35
MET-MET-SER-PHE-VAL-SER-LEU-LEU-LEU-VAL-GLY-ILE-LEU-PHE- . -ALA-THR-GLN-ALA-GLU-GLN-LEU-THR-LYS-CYS-GLU-VAL-PHE-GLN- . -LEU-LYS-ASP-LEU-LYS-

1 Hen's egg white pre-lysozyme(34)10              15          18 19                  25                30
MET-ARG-SER-LEU-LEU-ILE-LEU-VAL-LEU-CYS-PHE-----LEU-PRO-LEU-ALA-ALA-LEU-GLY-LYS-VAL-PHE-GLY-ARG-CYS-GLU-LEU-ALA-ALA-ALA-MET-LYS-ARG-HIS-GLY-
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Fig. 4. *Amino terminal sequence of ovine pre-α-lactalbumin. Comparison with those of human* [4] *and bovine* [3] *α-lactalbumins and hen's egg white pre-lyso zyme.* [34].
Residues written in *ITALICS* are those of the "signal" peptides. Unidentified residues are marked by a dot. The alignment shown here is that required for maximum homology. Homologous residues of both signals as well as those commor to the 4 authentic proteins have been underlined.
Gln, Arg and Trp residues occurring at position 10 in the polypeptide chain of bovine α-lactalbumin caracterize different genetic variants.
Residue 15 in the signal of ovine α-lactalbumin is probably a tryptophan.

protein owner to pass once only the endoplasmic reticulum border. Since lactose synthesis depends upon the interaction of α-lactalbumin with the galactosyl transferase present in the Golgi region, the transport of the "modifier protein" is a crucial stage. Conceivably, α-lactalbumin-like proteins might also exist in some specie: whose milk is devoid of lactose (33), but not be able to play their role of "modifier' simply because they lack the "signal" device necessary for their transfer to the Golgi region.

In a preceding communication (16) to which the reader is referred, we have already compared the "signals" of the six major ovine secretory lactopro-

teins including α-lactalbumin and discussed their essential features. In the present paper, it is of interest to compare the signal of ovine α-lactalbumin with that of lysozyme (34) since both proteins might have derived from a common ancestor (1). The optimal alignment of both signal sequences (Fig. 4) reveals a 30% homology (6 homologous residues in a total of 19) in contrast with the 37% homology found between bovine α-lactalbumin and hen's egg white lysozyme (35). Thus the "signal peptide" seems to have evolved at least as rapidly as the remaining part of the polypeptide chain. Since both the hydrophobicity and the occurrence of clustered hydrophobic residues have been preserved during the course of evolution, this suggests that they are the essential features relevant to the functional properties of the signal in the early steps of secretion.

In this respect, studies of the chemical evolution of the "signal peptides" of lactoproteins from various species not closely related might be a promising way to gain valuable information concerning the essential intrinsic properties of the signal. Sequence analyses of pre-pieces of lactoproteins from pig and rabbit species are now in progress.

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Footnote: *When this paper was being written, the partial amino acid sequence of the signal of rat α-lactalbumin was reported by Lingappa et al (36). The 19 residues long pre-piece starts with Met-Met and contains 3 leucyl residues at positions 7, 9 and 14. On the basis of these preliminary data, the signal of α-lactalbumin appears to be very similar in both rat and sheep species.*

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