Cell-free Synthesis of Rat Parotid Preamylase*

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Poly(A)-containing RNA from rat parotid gland directs the cell-free synthesis of several products in the reticulocyte lysate translation system including a very prominent 58,000-dalton polypeptide which is immunoreactive with anti-α-amylase. Purified α-amylase has a molecular weight estimated as 56,000 daltons. The 58,000-dalton, cell-free product and α-amylase share common peptides as determined by analysis of their limited proteolysis digests. The cross-reactivity and peptide homology suggest that the cell-free product may be a precursor of mature α-amylase. While the NH₂ terminus of α-amylase is blocked, that of the 58,000-dalton product evidently is not, and automated sequence analysis has yielded its partial sequence as: Met-X-Phe-Phe-Leu-Leu-X-Leu-X-Leu-X-X-X-X-x-X-Phe-X-X-X-X-X-Leu-X-Leu-Phe. The highly hydrophobic nature of the NH₂ terminus of the 58,000-dalton, cell-free product suggests that, like other secreted polypeptides, the extra piece may play a role in the transport and secretion of the mature α-amylase.

Rat parotid gland, highly specialized for the synthesis of a few proteins (1), provides a system amenable to study the secretion of enzymes. The major protein component in the gland is α-amylase which accounts for about 30% of the total proteinaceous content of its secretory granules. This protein, consisting of a single polypeptide chain with a molecular weight of 56,000 (2) was shown to be synthesized on membrane-bound polyribosomes of rough endoplasmic reticulum (3). The newly synthesized protein is transported from the rough microsomes to the smooth endoplasmic reticulum, then to the Golgi apparatus, and is finally sequestered in secretory vesicles (4, 5). Several other secretory proteins have been shown to be synthesized and secreted by analogous mechanisms (6, 7). The rate of secretion of α-amylase depends upon hormonal stimulation of the β adrenergic receptor and is triggered by catecholamines (8, 9).

Recent reports indicate that most secretory proteins are synthesized as precursors with a hydrophobic extension peptide at the NH₂ terminus. Cell-free translation of mRNA coding for immunoglobulins (Refs. 10 and 11, reviewed in Ref. 12), hormones (13-16), secretory proteins (17, 18), and secretory enzymes (19-21) yields products larger than the mature secretory proteins. The signal hypothesis formulated by Blobel and Dobberstein (22) postulates that a "signal peptide" located at the NH₂ terminus of these precursors participates in the binding and transport of nascent chains across the membrane of the rough endoplasmic reticulum.

We have investigated the synthesis of α-amylase in the rat parotid gland. This paper describes the isolation of mRNA from rat parotid coding for a polypeptide sharing common sequences with α-amylase as judged by immunoprecipitation with anti-α-amylase and peptide patterns. The primary translation product has a molecular weight larger than authentic α-amylase and the partial sequence of the isolated polypeptide reveals a cluster of hydrophobic amino acid residues at the NH₂ terminus. This indicates that the main cell-free translation product of rat parotid mRNA represents a precursor to α-amylase similar in its NH₂-terminal sequence to precursor of other secretory proteins previously described.

MATERIALS AND METHODS

[35S]Methionine (500 to 1000 Ci/mmole) and [4,5-3H]leucine (40 to 60 Ci/mmole) were from New England Nuclear. [2,3-3H]Isoleucine, [3H]phenylalanine, and [3H]valine (10 to 30 Ci/mmole) were purchased from Nuclear Research Center, Negev, Israel. Oligo(dT)-cellulose was a generous gift from Drs. I. Schechter and Y. Burstein and purified rabbit anti-goat immunoglobulin was from Dr. I. Schechter.

Animals—Sprague-Dawley or Louis rats weighing 200 to 300 g each were used. For mRNA isolation, the rats were fasted overnight, injected intraperitoneally with isoproterenol (1 mg/lOO g of animal weight) in PBS, and killed after 2 h by anestheic. The parotid glands were removed, washed with cold PBS, and stored in liquid nitrogen.

Isolation of RNA—The total RNA was extracted from frozen parotid glands by phenol/creosol method II of Kirby (23). Poly(A)-containing mRNA was purified by oligo(dT)-cellulose chromatography as described previously (24).

Labeling of α-Amylase with [35S]Methionine—Parotid glands (0.3 g) freshly removed from two rats fed ad libitum were minced, washed twice with Duibecoo's modified Eagle's medium containing all amino acids except methionine, and finally suspended in 2 ml of the same medium supplemented with 50 μCi of [35S]methionine. After incubation for 1 h at 37°C, the tissue was removed, washed three times with PBS, homogenized in a Dounce homogenizer, and centrifuged at 100,000 x g for 60 min. The main labeled polypeptide in the postmicrosomal extract was α-amylase (Fig. 1c).

Preparation of Goat Antiamylase—Antibodies against purified homologous α-amylase (5B) were prepared in the goat and purified by immunoabsorption (26) on an α-amylase-Sepharose column.

Cell-free Protein Synthesis—Rabbit reticulocyte lysates were prepared and treated with micrococcal nucleolus according to the procedure of Pelham and Jackson (27). Reaction mixtures (25 μl) contained: 0.25 mM magnesium acetate, 500 μM spermidine (free base), 5 mM creatine phosphate (P-L Biochemicals), 10 μg/ml of creatine kinase (Sigma), 94 mM potassium acetate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) at pH 7.6, 2 mM dithiothreitol, 0.25 mM magnesium acetate, 500 μM spermidine (free base), 8 mM sodium dodecyl sulfate; NaDodSO₄, sodium dodecyl sulfate. The abbreviations used are: PBS, 10 mM sodium phosphate (pH 7.4), 0.15 M sodium chloride; NaDODSO₄, sodium dodecyl sulfate.
The enrichment for poly(A)-containing RNA was achieved by two subsequent cycles of oligo(dT)-cellulose chromatography. Twice bound poly(A)-RNA represented approximately 1.7% of the total RNA.

**RESULTS**

**Isolation of mRNA**—Analytical immunoprecipitation of rat parotid polysomes with antiamylase had indicated that the level of mRNA specific for α-amylase is augmented approximately 10-fold when fasted rats were stimulated by injection of catecholamines, as compared to normal or fasted rats. Preliminary attempts to extract the RNA from the isolated polysomes were unsuccessful. Rat parotid gland contains a considerable level of RNase (25, 30) which poses a serious obstacle to the isolation of an intact RNA. In the intact cell, however, the compartmentalization of the cytoplasm possibly limits the damage wrought by RNase. It was found that the method of Kirby (23) was very efficient in overcoming the effect of RNase. Accordingly, the intact, frozen parotid glands from rats injected with isoproterenol or isoprenaline were thawed directly into the denaturative medium and total RNA was extracted by the phenol/cresol method.

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**Translation of mRNA** and **Nature of Cell-free Products**—Addition of total rat parotid poly(A)-RNA to mRNA-dependent rabbit reticulocyte lysate cell-free systems resulted in 3- to 25-fold stimulation of the radioactive amino acids incorporation into proteins, depending upon the labeled amino acids used. The [35S]methionine-labeled cell-free products were fractionated on NaDodSO4/polyacrylamide slab gels and analyzed by autoradiography. One of the most prominent polypeptides synthesized in the system was that with a molecular weight of approximately 58,000 (Fig. 1b). Only this polypeptide was specifically immunoprecipitated from the total cell-free products by sequential treatment with goat antiserum prepared against purified α-amylase, followed by addition of purified rabbit anti-α-amylase (Fig. 1d). Authentic α-amylase migrates somewhat faster in this system (Fig. 1c), giving an expected molecular weight of 56,000 (2) (Fig. 2). However, it should be remembered that molecular weight determination based on NaDodSO4/polyacrylamide gel electrophoresis are prone to error, especially when one is dealing with glycoproteins, which show a misleadingly high apparent molecular weight. Therefore, the difference in molecular weights between the cell-free corresponding product and α-amylase, which is glycoprotein (2), may be even greater.

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FIG. 3. Fluorogram of the cleavage products of α-amylase (a to e) and preamylase synthesized in vitro (f to j). Protein digest analyses were performed as described under “Materials and Methods.” Tracks a and f, no protease added; Tracks b, c, and g, h, with 5 μg/ml and with 2 μg/ml of chymotrypsin, respectively; Tracks d, e, and k, l, with 2.5 and 1 μg/ml of papain, respectively.

FIG. 4. Radioactivity recovered at each sequenator cycle from the cell-free products immunoprecipitated with antiamylase as described under “Materials and Methods.” The products were labeled with: A, [35S]methionine (15,000 cpm); B, [3H]phenylalanine (39,000 cpm); C, [3H]isoleucine (22,000 cpm). Cycle zero represents a blank cycle (without phenyl isocyanate) which was used to wash out potential radioactive contaminants.

Peptide Analysis—The relationship between authentic α-amylase and cell-free product immunoprecipitated with antiamylase was further investigated by partial proteolytic digestion. The [35S]methionine-labeled α-amylase and 58,000-dalton cell-free product were eluted from NaDodSO₄/polyacrylamide gels (Fig. 3, a and f) and after limited digestion with chymotrypsin or papain, the digests were resolved on NaDodSO₄/15% polyacrylamide gels. Autoradiograms show that with papain the in vivo (Fig. 3, d and e) and in vitro (Fig. 3, i and j) peptide patterns are identical. Limited digestion with chymotrypsin revealed two identical peptides and an additional one which migrates faster in α-amylase (Fig. 3, b, c and g, h). This peptide probably represents the NH₂-terminal peptide of α-amylase with the extension of an “extra piece.”

Based on the above data, it was concluded that the 58,000-dalton polypeptide in vitro product was identical to α-amylase but contained an additional polypeptide chain of approximately 2,000 daltons and may be alluded to as the preamylase.

Sequence Analyses—To gain more information on the nature of putative preamylase, a partial amino acid sequence of the cell-free products labeled with radioactive amino acids and indirectly immunoprecipitated with antiamylase was determined by automatic Edman degradation. Figs. 3 and 4 show that the release of peaks of radioactivity is associated with certain cycles of Edman degradation of the immunoprecipitated cell-free products. Radioactive peak from the product labeled with [35S]methionine occurs only at Cycle 1 (Fig. 4A); with [3H]phenylalanine-labeled product the peaks are at Cycles 3, 4, and 22 (Fig. 4B); with [3H]isoleucine-labeled product the peaks appear at Cycles 10 and 28 (Fig. 4C); products labeled with [3H]leucine show radioactive peaks at positions 5, 6, 7, 9, 12, 31, and 48 (Fig. 4D). Flat and low background of radioactivity was obtained from sequencer runs (30 cycles) of precursor labeled with [3H]valine, thus showing that this amino acid is not present in the preamylase (not shown). Discrete radioactive peaks were recovered from Sequencer runs of the labeled precursor. In the semilog plot (Fig. 4B), the peaks lay on a straight line, thus showing that they originated from one protein species. The results, sum-
marized in Fig. 6, illustrate the partial sequence of first 35 amino acids of putative precursor to α-amylase.

**DISCUSSION**

This report shows that partially purified rat parotid mRNA directs the *in vitro* synthesis of several distinct polypeptides in a reticulocyte lysate cell-free protein synthesizing system. A considerable fraction of the polypeptide material produced in this system was specifically immunoprecipitated with antiamylase. The amount of selective immunoprecipitate varied from 8 to 30% of the total products synthesized, depending on amino acid used for labeling. The immunoprecipitated material appeared to be homogeneous in size with a molecular weight of about 58,000 which is larger than purified rat parotid α-amylase by 2,000. It was shown by means of limited proteolysis, in NaDodSO₄-containing buffer, that the peptide patterns generated independently by two proteases with different specificities are almost identical for authentic amylase and the corresponding polypeptide synthesized *in vitro*.

On the basis of this evidence, it is concluded that the immediate translation product of parotid amylase mRNA is a polypeptide somewhat larger than the *in vivo* secreted, mature α-amylase and will be referred to as preamylase. The fidelity of translation of several mRNAs in the reticulocyte lysate system has been demonstrated. In most cases the products have an NH₂-terminal methionine residue (18, 20, 32, 33), which has been identified as the initiator methionine (20, 32, 34). Sequence analysis of preamylase reveals a methionine residue at Cycle 1, suggesting that it is the initial translation product of mRNA.

Parotid amylase has a blocked NH₂ terminus and its primary structure has not been published. At the moment, therefore, it is not possible to align the amino acid sequences of α-amylase and preamylase and determine the exact size of the extra piece. The partial sequence of preamylase reveals a cluster of hydrophobic amino acid residues at the NH₂ terminus; the region within positions 3 to 12 encompasses hydrophobic residues with the sequence Phe-Phe-Leu-Leu-X-Leu-Ile-X-Leu-X-X-X-X-X-X-X-X-Phe-X-X-X-X-Ile-X-X-Leu-Phe. As emphasized by Schechter and Burstein (33), this structural feature is characteristic of the extra piece found *in vitro* on the translation product of messenger RNAs coding for a variety of other secretory proteins. It is generally accepted that secretory proteins are synthesized on microsomes. According to signal hypothesis (22), the amino acid extension of 15 to 30 amino acid residues at the NH₂ terminus (signal peptide) is recognized by membrane receptors and directs the polypeptide into the endoplasmic reticulum. The growing nascent chains are voclariaally discharged across the microsome membrane to the Golgi area and stored in secretory granules.

Transit signal peptides associated with preproteins have been postulated to be rapidly cleaved by microsomal proteases (10, 35, 36) during each cycle of protein synthesis, while the exported protein is matured on the other side of the membrane. The processing of preproteins has recently been shown in different systems (10, 20, 34, 35, 36). However, preliminary experiments in our laboratory to demonstrate the conversion of preamylase to amylase using rat parotid microsomes added co-translationally, as the source of specific protease, were unsuccessful. This was probably due to the presence of ribonuclease in the preparation which hampered translation. As yet, only the protease from rough microsomes of dog pancreas, having a rather broad specificity towards preproteins, could be solubilized with aid of detergents (37). We are currently investigating the use of this system to accomplish preamylase maturation *in vitro*. Amylase obtained from cleavage of preamylase in a cell-free system devoid of blocking activity, may have a free NH₂ terminus and could then be sequenced providing additional evidences for the size of the extra piece.

The *in vitro* isolation and translation of the mRNA coding for dog pancreatic preamylase has been recently reported (21). Pancreatic amylase, was previously shown to be completely unrelated to parotid enzyme by lack of immunological cross-reactivity, different amino acid composition (2) and different peptides pattern (38), although both enzymes possesses the same enzymatic activity. It was, therefore, suggested that the two enzymes are different unique molecules and thus the product of different genes. The primary sequence of pancreatic amylase has not been established. It will be of interest to compare these two unrelated amylases and to ascertain whether there is homology in their presequences.

Preamylase synthesized *in vitro* does not display any enzymatic activity for hydrolysis of starch. This activity, if it existed, would most likely be detectable, as corresponding amounts of amylase do show starch hydrolysis (data not shown). The absence of biological activity may well be due to the extra piece. The hydrophobic extension at the NH₂ terminus can prevent the proper folding of the preprotein, which is prerequisite for acquiring the full biological activity (39). The short lived extra piece is removed prior to maturation, liberating the biologically active molecule in the native conformation. Such a mechanism may play an important role in control of the secretion of enzymes.

**Acknowledgments—**We warmly thank Dr. I. Schechter for stimulating discussions and encouragement throughout this work as well as Drs. Y. Burstein and C. Prives for their critical reading of the manuscript. Mrs. Z. Avnur is thanked for her excellent technical help.

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*Unpublished observation.*
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J. Biol. Chem. 1979, 254:525-529.

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