Warren, G., Houslay, M., Metcalfe, J., & Birdsall, N. (1975) *Nature (London) 255,* 684.
Wolf, D., Schlessinger, J., Elson, E., Webb, W., Blumenthal, R., & Henkart, P. (1977) *Biochemistry 16,* 3476.
Wu, E.-S., Jacobson, K., & Papahadjopoulos, D. (1977) *Biochemistry 16,* 3936.
Wu, E.-S., Szoka, F., & Jacobson, K. (1978) *Biophys. J. 21,* 214a.

Conversion of Pre-Proparathyroid Hormone to Proparathyroid Hormone by Dog Pancreatic Microsomes†

Andrew J. Dorner and Byron Kemper*

ABSTRACT: In the wheat germ extract and reticulocyte lysate cell-free protein synthesizing systems, the major translation product of bovine parathyroid hormone mRNA was a precursor of parathyroid hormone, pre-proparathyroid hormone and little or no proparathyroid hormone or parathyroid hormone was produced. If dog pancreatic rough microsomes were added to the wheat germ system, a new radioactive protein was synthesized which coelectrophoresed with proparathyroid hormone on acrylamide gels containing sodium dodecyl sulfate and urea–acid acrylamide gels at pH 4. Analysis by Edman degradation of this protein labeled with [^{3}H]lysine revealed lysines at positions 1, 4, and 5 as expected for authentic proparathyroid hormone. Conversion of pre-proparathyroid hormone to proparathyroid hormone occurred only if the membranes were present while pre-proparathyroid hormone was being synthesized. Inhibition of the conversion activity by a nonionic detergent, Nonidet P40, suggested that the proteolytic activity was associated with the membranes. Radioactive proparathyroid hormone but not pre-proparathyroid hormone was protected from digestion by proteolytic enzymes added posttranslationally to the reactions that contained membranes suggesting that proparathyroid hormone had been transported across the microsomal membrane into the lumen of the vesicles. Although analyzed less extensively, the addition of microsomal membranes to the reticulocyte lysate cell-free system is also required for the synthesis of proparathyroid hormone. The fraction of pre-proparathyroid hormone that is converted to proparathyroid hormone in the reticulocyte lysate is about two times that observed in the wheat germ cell-free system. These data demonstrate that, in completely heterologous cell-free systems, pre-proparathyroid hormone can be accurately processed to form proparathyroid hormone by a proteolytic activity associated with microsomal membranes.

In the wheat germ cell-free protein synthesizing system the translational product of parathyroid hormone (PTH)[1] mRNA is pre-proparathyroid hormone (pre-ProPTH) which contains the sequence of PTH and 31 additional amino acids at the N terminus (Kemper et al., 1974, 1976). Pre-ProPTH is essentially the only form of PTH synthesized in cell-free systems that do not contain microsomal membranes, such as the wheat germ system. In intact cells pre-ProPTH is detected in tiny quantities (Habener et al., 1976) and is presumably rapidly converted to proparathyroid hormone (ProPTH) by proteolytic cleavage of 25 N-terminal amino acids. ProPTH is an intracellular precursor which is then converted to PTH by proteolytic cleavage of 6 N-terminal amino acids about 15 min after initial synthesis (Kemper et al., 1972; Cohn et al., 1972). The processing of these larger forms of PTH thus requires two very specific proteolytic cleavages only 6 amino acids apart.

Larger pre-proteins analogous to pre-ProPTH have now been described for many secretory proteins. It has been proposed that the extra sequences at the N terminus (pre-sequence) of these proteins function in the formation of membrane-bound ribosomes and the transport of the secretory proteins through the membrane of the endoplasmic reticulum (Blobel & Sabatini, 1971; Milstein et al., 1972; Blobel & Dobberstein, 1975). The removal of the pre-sequence then would be mediated by enzymes associated with these membranes. In cell-free systems, cleavage of the pre-sequence has been shown to be dependent on the addition of microsomal membrane preparations for myeloma protein (Blobel & Dobberstein, 1975), human placental lactogen (Boime et al., 1977), insulin (Shields & Blobel, 1977), prolactin and growth hormone (Lingappa et al., 1977). The translation of PTH mRNA in the unfractionated Krebs II ascites cell-free system which contains microsomal membranes results in the synthesis of ProPTH as well as pre-ProPTH (Habener et al., 1975). In this paper we show that accurate conversion of pre-ProPTH to ProPTH occurs in the wheat germ and reticulocyte cell-free systems if microsomal membranes from dog pancreas are added to the reaction.

Experimental Procedures

Preparation of Stripped Microsomes. Fresh dog pancreases were chilled and rinsed with 0.9% NaCl and fibrous tissue was removed manually. Five grams of pancreatic tissue in 8 mL of 0.25 M sucrose, 50 mM triethanolamine, pH 7.4 (20 °C), 50 mM KCl, and 5 mM $MgCl_2$ was homogenized with four strokes in a motor driven glass–Teflon Potter-Elvehjen tissue grinder at 150–200 rpm. A postmitochondrial supernatant was prepared by centrifuging the tissue homogenate at 10 000*g* for

† From the Department of Physiology and Biophysics and School of Basic Medical Sciences-UC, University of Illinois, Urbana, Illinois 61801. *Received June 8, 1978.* This work was supported by a grant from the National Institutes of Health.

[1] Abbreviations used: PTH, parathyroid hormone; ProPTH, proparathyroid hormone; pre-ProPTH, pre-proparathyroid hormone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/78/0417-5550$01.00/0

10 min at 5 °C. Rough microsomes were isolated from the postmitochondrial supernatant by discontinuous sucrose gradient centrifugation, ribosomes on the membranes were released or inactivated by treatment with EDTA, and the resulting stripped rough microsomes were isolated by centrifugation on a 10–55% sucrose gradient essentially as described by Blobel & Dobberstein (1975), with modifications described below. A Beckman type 65 rotor replaced the IEC type A-211 rotor and volumes were reduced by a factor of 3 and a Beckman SW41 rotor replaced the IEC SB283 rotor. The turbid band corresponding to the stripped rough microsomes in the 10–55% gradient was removed with a syringe. For cell-free protein synthesis, the stripped rough microsomes were resuspended by gentle homogenization in a Teflon–glass homogenizer in 0.25 M sucrose, 100 mM KCl, 20 mM Hepes·KOH, pH 7.3 (20 °C), 3 mM $MgCl_2$, and 2 mM dithiothreitol to a concentration of about 115 A_{260} units/mL and the suspension was sonicated.

Cell-Free Protein Synthesis. Messenger RNA, referred to as PTH mRNA, used in these experiments was bovine parathyroid RNA sedimenting between approximately 8 and 15 S and was isolated as described previously (Kemper, 1976). Conditions for protein synthesis in the wheat germ system were as described previously (Kemper et al., 1974) except that 2 mM $MgCl_2$ instead of 3 mM $MgCl_2$ was used because, in reactions containing stripped rough microsomes, 2 mM $MgCl_2$ was optimal for protein synthesis. Maximal synthesis of ProPTH occurred when stripped rough microsomes were added to the reactions in a concentration that reduced incorporation of [^{35}S]methionine into protein by 50%. The relative efficiency of ProPTH synthesis was not significantly changed by altering the $MgCl_2$ or KCl concentrations in the reaction mixture. The amount of ProPTH synthesized increased for up to 2 h of incubation. Lysates of reticulocytes obtained from phenylhydrazine-treated rabbits were prepared as described by McDowell et al. (1972). Reticulocyte lysates were treated with micrococcus nuclease as described by Pelham & Jackson (1976). The final reaction contained in a volume of 25 μL: 10 μL of reticulocyte lysate, 0.6 mM $MgCl_2$, 120 mM KCl, 0.5 mM spermidine, 8.8 mM phosphocreatine, 80 μg/mL phosphocreatine kinase, and 25 mM Hepes·KOH, pH 7.4.

Total radioactivity incorporated into protein was assayed by determining the amount of trichloroacetic acid-insoluble radioactivity in 1-μL aliquots as described previously (Kemper et al., 1974). The radioactive translational products were analyzed as described previously on slab 15% acrylamide gels containing sodium dodecyl sulfate (Kemper, 1976) and on cylindrical 10% acrylamide gels containing 8 M urea at pH 4 (Kemper et al., 1972).

Sequence Analysis of ProPTH. Radioactive ProPTH was isolated from acid–urea acrylamide gels as described previously (Kemper et al., 1972) except that calf thymus histones were used as a carrier instead of a partially purified preparation of PTH. Sequential degradation was performed as described by Gray (1967). After cleavage of the phenylthiocarbamoyl peptide and removal of trifluoroacetic acid, the sample was dissolved in water and the relased amino acid extracted with *n*-butyl acetate. Radioactivity was assayed by adding 2 mL of the *n*-butyl acetate extract to 9 mL of toluene scintillation fluid (4 g/L 2,5-phenyloxazole and 0.1 g/L 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene) and adding 2 drops of NCS solubilizer (Amersham/Searle Corp.) to clarify the solution.

Proteolysis of Translational Products. The resistance of the translational products to proteolytic enzymes was assayed as described by Shields & Blobel (1977) with modifications. After an incubation of 3 h at 23 °C, the wheat germ reactions were incubated with 250 μg/mL of chymotrypsin for 30 min at 28 °C. In some experiments 250 μg/mL of trypsin was also added.

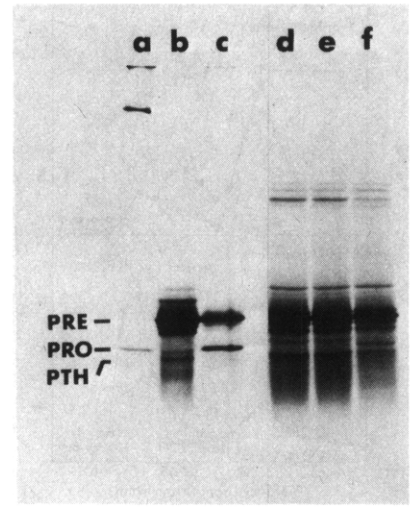

FIGURE 1: Analysis by electrophoresis of the translation products of PTH mRNA in the presence and absence of stripped rough microsomes. PTH mRNA was translated in the wheat germ system. The radioactive proteins were electrophoresed on 15% acrylamide slab gels containing sodium dodecyl sulfate and fluorograms (slots a–c) or autoradiograms (slots d–f) were prepared by exposure to the film for 3.5 and 2 days, respectively. Proteins were labeled with [^{3}H]lysine in slots b and c and [^{35}S]methionine in slots d–f. The positions of pre-ProPTH (PRE), ProPTH (PRO), and PTH are indicated and were determined by analysis of [^{35}S]methionine-labeled proteins extracted from parathyroid tissue, parathyroid secretory protein (upper band), and ProPTH (lower band) shown in slot a; (slot b) PTH mRNA added; (slot c) PTH mRNA plus stripped rough microsomes (17 A_{260} units/mL) added; (slot d) PTH mRNA added to a reaction incubated for 90 min; (slot e) PTH mRNA added to a reaction incubated for 90 min after which stripped rough microsomes and 1 mM 7-methylguanosine 5′-phosphate were added during an additional 90-min incubation; (slot f) PTH mRNA plus stripped rough microsomal membranes incubated for 90 min.

Results

In previous studies we have shown that the major translation product of bovine PTH mRNA in the wheat germ system is pre-ProPTH (Kemper et al., 1974). As shown in Figure 1b, the synthesis of ProPTH in this system could not be detected when the translation products were analyzed by electrophoresis on acrylamide gels containing sodium dodecyl sulfate. In contrast, if stripped rough microsomes were added to the reaction, a new radioactive protein that comigrated with ProPTH was observed (Figure 1c). If the translation products were analyzed by electrophoresis in acrylamide gels containing 8 M urea at pH 4 (urea–acid gels), little or no synthesis of proteins migrating with ProPTH was observed unless the stripped rough microsomes were added to the reaction (Figure 2). Pre-ProPTH was detected with an efficiency of only 10–20% on these acid–urea acrylamide gels compared with those containing sodium dodecyl sulfate and thus the apparent extent of conversion is misleading (compare Figure 1c with Figure 2). The reason for the consistent low recovery of radioactive pre-ProPTH on the acid–urea acrylamide gels is not known but pre-ProPTH isolated from sodium dodecyl sulfate–acrylamide gels appears homogeneous by tryptic peptide and sequence analysis (Kemper et al., 1976) and thus the discrepancy is not explained by contaminating proteins that comigrate with pre-ProPTH in the sodium dodecyl sulfate gels. If [^{35}S]methionine instead of [^{3}H]lysine was used as the radioactive label, the amount of the radioactive new protein was decreased relative to the

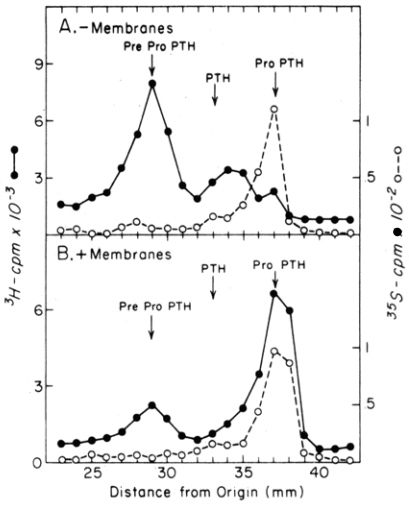

FIGURE 2: Analysis by electrophoresis on acid–urea acrylamide gels of the translation products of PTH mRNA in the presence and absence of membranes. Reaction conditions with [^{3}H]lysine as label were as described in the legend to Figure 1. Translation products were electrophoresed on 10% cylindrical acrylamide gels at pH 4 in the presence of 8 M urea. The gels were sliced in 1-mm sections and radioactivity was determined in each slice. Only the regions of the gel containing the PTH-related proteins are shown. The relative positions of ^{35}S-labeled ProPTH and PTH analyzed on the same gels are indicated. (Panel A) PTH mRNA added to the reaction; (panel B) PTH mRNA plus stripped rough microsomes (17 A_{260} units/mL) added to the reaction.

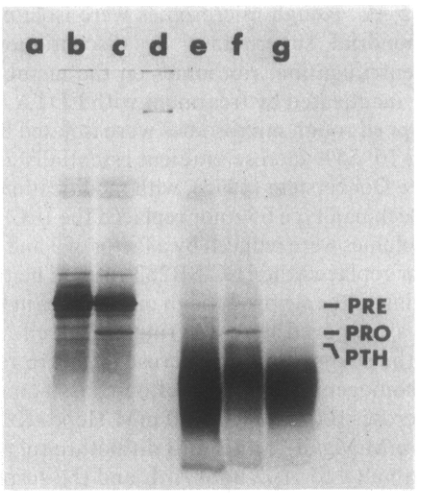

FIGURE 3: Resistance of ProPTH to proteolytic digestion of chymotrypsin. PTH mRNA was translated in the wheat germ system and reactions with membranes contained 17 A_{260} units/mL. The ^{35}S-labeled proteins were electrophoresed on 15% acrylamide slab gels containing sodium dodecyl sulfate, and autoradiograms of the dried gel were produced by 4.5-day exposure to the film. The positions of Pre-ProPTH (PRE), ProPTH (PRO), and PTH are indicated. (Slot a) Minus mRNA; (slot b) PTH mRNA: (slot c) PTH mRNA plus membranes; (slot d) [^{35}S]-methionine-labeled parathyroid secretory protein (upper band) and ProPTH (lower band); (slot e) as in slot b after treatment with 250 μg/mL chymotrypsin; (slot f) as in slot c after treatment with 250 μg/mL chymotrypsin; (slot g) same as slot f except 0.5% deoxycholate was added during the incubation with chymotrypsin.

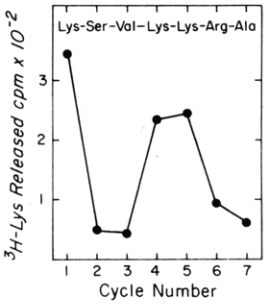

FIGURE 4: Analysis by Edman degradation of ProPTH synthesized in wheat germ extracts containing stripped rough microsomes. Cell-free translational products labeled with [^{3}H]lysine were electrophoresed on acid-urea acrylamide gels as in Figure 2, and radioactive protein corresponding to ProPTH was extracted from the gel. The isolated protein was subjected to manual sequential Edman degradation, and the amount of [^{3}H]lysine released after each cycle was assayed. The sequence of the first 7 N-terminal amino acids of ProPTH is shown in the figure.

amount of pre-ProPTH (Figures 1f and 3c). This would be expected if the new protein is ProPTH, since ProPTH contains only 2 of the 7 methionines but 12 of the 14 lysines in pre-ProPTH (Kemper et al., 1976; Hamilton et al., 1972; Jacobs et al., 1974).

To prove that the new protein synthesized in the presence of the microsomal membranes was ProPTH and to assess the accuracy of the proteolytic cleavage, we isolated the [^{3}H]lysine-labeled protein from the acid–urea acrylamide gels and analyzed its sequence by sequential Edman degradation. As shown in Figure 4, radioactivity was released at steps 1, 4, and 5 which correspond to the positions of lysine in ProPTH (Hamilton et al., 1972; Jacobs et al., 1974). Proteolytic activity present in the stripped rough microsomes was, therefore, able to accurately process pre-ProPTH to produce ProPTH. At the same time synthesis of PTH could not be detected, further emphasizing the specificity of the reaction since the -Arg-Ala- bond cleaved to convert ProPTH to PTH is extremely sensitive to trypsin-like enzymes (Cohn et al., 1972; Goltzman et al., 1976).

The autoradiograms shown in Figures 1c, 1f, and 3c suggest that the 25 amino acid pre-sequence that is cleaved from pre-ProPTH was not recovered intact after the reaction. This small piece should contain 5 methionines compared with only 2 methionines in ProPTH and thus should contain 2.5 times as much radioactivity. There was no suggestion of an additional band of radioactivity near the bottom of the gel in the presence of membranes. The reactions are simply mixed with a gel buffer and applied directly to the acrylamide gel so the small fragments would not be lost by manipulation of the product. A synthetic analogue of the pre-sequence of pre-ProPTH was not extracted from similar gels by the staining and destaining procedures (J. F. Habener, personal communication). Furthermore, we were not able to detect the pre-sequence on two other gel electrophoresis systems: cylindrical 15% acrylamide gels containing 8 M urea and 0.1% sodium dodecyl sulfate and slab 17.5% acrylamide gels containing 8 M urea and 0.1% $NaDodSO_4$. These data suggest either that the pre-sequence is removed by exoproteolysis or that it is digested rapidly after cleavage.

The conversion of pre-ProPTH to ProPTH mediated by the microsomes occurred only if the microsomes were present during the translation of the mRNA. If they were added after 90 min of incubation and further initiation of protein synthesis was blocked by the addition of 7-methylguanosine 5′-phosphate (Hickey et al., 1976), the radioactive products were not detectably different from a reaction for 90 min without microsomal membranes (Figures 1d and 1e). If membranes were present during the initial 90 min of incubation, ProPTH was synthesized as expected (Figure 1f).

To examine whether pre-ProPTH was not only processed to ProPTH but was also sequestered within the membrane vesicles after processing, we examined the relative resistance of the translational products to proteolytic digestion. After an

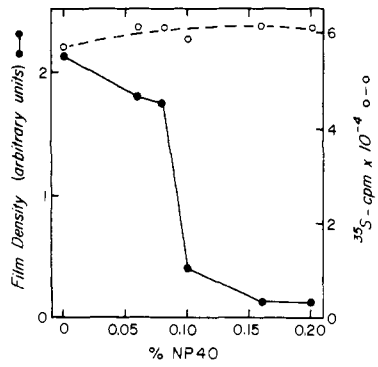

FIGURE 5: Inhibition of the synthesis of ProPTH by Nonidet P40. PTH mRNA was translated in the presence of rough stripped membranes (14 A_{260} units/mL) in the wheat germ system. Increasing amounts of Nonidet P40 (NP40) were added to reactions as indicated. Reaction products were analyzed on slab gels as described in Figure 1. The amount of ProPTH synthesized was estimated by densitometric scans of autoradiograms of the gels. (O - - O) Total incorporation of [^{35}S]methionine into protein per μL of reaction; (●—●) ProPTH synthesized.

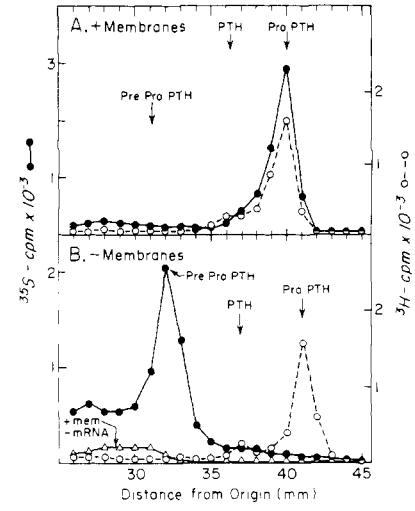

FIGURE 6: Conversion of pre-ProPTH to ProPTH in the reticulocyte lysate cell-free system. Translational products were analyzed by electrophoresis on acid-urea acrylamide gels as in Figure 2. (●—●) PTH mRNA was translated in nuclease-treated reticulocyte lysates in the absence (panel B) or presence (panel A) of stripped rough microsomes (6 A_{260} units/mL) with [^{35}S]methionine as label. (Δ—Δ) Reactions contained stripped rough microsomes but no PTH mRNA. (O - - O) Proteins were extracted from parathyroid tissue after incubation with [^{3}H]leucine. ProPTH is the major protein that is labeled.

incubation of 3 h, chymotrypsin was added to the reaction. In reactions containing microsomal membranes, pre-ProPTH was almost completely digested to smaller products while a substantial fraction of intact ProPTH remained (Figure 3f). No radioactive band corresponding to ProPTH was present after chymotrypsin digestion of reactions not containing membranes (Figure 3e). In addition, if 0.5% deoxycholate was added to the membrane-containing reactions during the digestion with chymotrypsin, no ProPTH remained (Figure 3g). These experiments strongly suggest that the ProPTH synthesized was sequestered within the lumen of the microsomal vesicles and thus protected from the proteolytic enzyme. The protection was not absolute since the amount of radioactivity in ProPTH was reduced. In addition, if trypsin was combined with chymotrypsin, ProPTH was still resistant to the digestion relative to pre-ProPTH but less ProPTH was recovered and more radioactivity was recovered in a diffuse band, the upper part of which comigrated with PTH (not shown). This might be expected since, as noted above, conversion of ProPTH to PTH is catalyzed by low concentrations of trypsin.

To examine whether the membrane portion of the stripped microsomes were involved in the conversion of pre-ProPTH to ProPTH, we studied the effects of the nonionic detergent, Nonidet P40, on the conversion process. Blobel & Dobberstein (1975) and Boime et al. (1977) have reported that nonionic detergents inhibit the cleavage of pre-proteins in cell-free systems. The addition of the detergent up to concentrations of 0.2% had minimal effects on the total protein synthesis in the wheat germ system (Figure 5). In contrast, between 0.08% and 0.1% Nonidet P40 the conversion of pre-ProPTH to ProPTH was sharply inhibited, and at a detergent concentration of 0.2% less than 5% of ProPTH was synthesized compared with the control. Lower quantities of detergent affect the membrane properties. Light scattering by the membranes measured at 450 nm was reduced by more than 75% by 0.06% Nonidet P40 and visible banding of the membranes on 10–55% sucrose gradients was not observed if they were treated with 0.05% to 0.08% Nonidet P40 (data not shown). It is possible that the inhibition of conversion at the higher concentrations of Nonidet P40 resulted from more direct effects on the enzyme rather than the gross disruption of the membrane structure. Nevertheless, the inhibition of the conversion by a detergent suggests that the lipid portion as well as the proteins of the membrane is involved in the overall conversion process.

Only a fraction of the pre-ProPTH was converted to ProPTH by the microsomal membranes added to the wheat germ system. To establish the relative efficiency of conversion we cut out the bands corresponding to pre-ProPTH and ProPTH from the gel shown in Figure 1c. Thirty percent of the total radioactivity (corrected for two less lysines in ProPTH) in the two bands was present in ProPTH. It is possible that the relatively low efficiency of the conversion of pre-ProPTH to ProPTH observed in the wheat germ system was because of the disparate sources of ribosomes and membranes from a plant and a mammal, respectively. If the PTH mRNA was translated in a mRNA-dependent reticulocyte lysate cell-free system, ProPTH was again synthesized only if the stripped rough membranes were added to the reactions (Figure 6). The translational products of the reticulocyte lysate could not be analyzed on the slab acrylamide gels containing sodium dodecyl sulfate because pre-ProPTH and ProPTH migrated near the large amount of globin present in the lysate, and the radioactive bands corresponding to them were smeared and could not be resolved. To quantitate conversion in the reticulocyte system, translational products were analyzed by electrophoresis on cylindrical acrylamide gels under two different conditions and the amount of radioactivity in 1-mm slices of the gels was determined. If analyzed on acrylamide gels containing sodium dodecyl sulfate, both pre-ProPTH and ProPTH are detected efficiently but are not resolved from each other (Kemper et al., 1974). One aliquot of the reaction mixture was analyzed on these gels to estimate the total amount of radioactivity in ProPTH and pre-ProPTH. If analyzed on urea–acid gels ProPTH is easily resolved from pre-ProPTH (Kemper et al., 1974), although the latter is not detected efficiently. A second aliquot of the reaction mixture, thus, was analyzed on these gels as in Figure 6 to determine the amount of radioactivity in ProPTH. Using this technique we estimated that 65–70% of the molecules of pre-ProPTH synthesized in the reticulocyte system were converted to ProPTH compared with only 30% in the wheat germ system. The explanation for the greater conversion in the reticulocyte lysate is not known but may be related to the fact that protein synthesis is more efficient in the reticulocyte system than the wheat germ system (Pehlam &

Jackson, 1976). Larger polysomes should form in the reticulocyte system resulting in an enhanced cooperative binding of ribosomes to membranes. Alternatively, there is ample evidence that an interaction between the 60S subunit and membrane is involved in binding the ribosomes to the membrane (Sabatini et al., 1966, Rosbach & Penman, 1971, Harrison et al., 1974). If this binding is required for optimal transport and processing of the pre-ProPTH, then plant ribosomes probably form less effective complexes with mammalian microsomes than mammalian ribosomes do.

Discussion

These studies demonstrate that pre-ProPTH can be accurately cleaved to produce ProPTH in a completely heterologous cell-free system. The cleavage is dependent on the addition of membranes from dog microsomes and can be inhibited by a nonionic detergent which presumably disrupts the membrane. The cleavage does not occur if the membranes are added posttranslationally and the newly formed ProPTH is resistant to proteolytic cleavage. The characteristics of the reaction, thus are similar to those reported for the in vitro cleavage of other pre-proteins (Blobel & Dobberstein, 1975; Boime et al., 1977; Birken et al., 1977; Shields & Blobel, 1977; Lingappa et al., 1977). After removal of the pre-sequence, pre-ProPTH undergoes a second cleavage in vivo to produce the final mature protein, PTH. This second cleavage presumably occurs in a second membranous organelle, the Golgi apparatus (Chu et al., 1974; Habener et al., 1977a). Our sequencing data demonstrate that ProPTH is intact after the cell-free microsomal-dependent cleavage of pre-ProPTH. Therefore, the first of these sequential cleavages can be accurately reproduced in this cell-free system without any detectable proteolytic activity corresponding to the second cleavage to produce PTH. In contrast, all subcellular fractions of bovine parathyroid tissue convert ProPTH to PTH in cell-free systems (MacGregor et al., 1976; Habener et al., 1977b). The presence of proteolytic activity in the microsomal membranes that converts pre-ProPTH to ProPTH is consistent with the suggestion that the pre-sequence of pre-ProPTH is involved in the formation of membrane-bound ribosomes and the transport of the ProPTH across the membranes of the endoplasmic reticulum (Kemper et al., 1974; Habener et al., 1976) and the hypothesis that the pre-sequences function, generally, in this manner for secretory proteins (Blobel & Sabatini, 1971; Blobel & Dobberstein, 1975; Milstein et al., 1972).

The capability of dog pancreatic microsomal membranes to cleave accurately pre-proteins from different tissues and different species is remarkable. In addition to bovine pre-ProPTH reported here, dog membranes have been reported to cleave bovine pre-prolactin and pre-growth hormone (Lingappa et al., 1977) and fish (sea raven) pre-proinsulin (Shields & Blobel, 1977) in the wheat germ system and mouse pre-myeloma light chain proteins using rabbit reticulocyte ribosomes (Blobel & Dobberstein, 1975). A membrane fraction from Krebs II ascites cells has been reported to process human pre-placental lactogen (Boime et al., 1977). The pre-sequences of these proteins, while generally hydrophobic in nature, have different lengths and in some cases the primary sequence at the cleavage site is different. In the myeloma protein, cleavage occurs between cysteine and aspartic acid (Burstein & Schechter, 1977), and in pre-ProPTH it occurs between glycine and lysine (Habener et al., 1978). The idea that the pancreatic microsomes contain several enzymes which are specific for proteins from different tissues does not seem likely. It is possible that interaction of the peptide with the membrane may be required for accurate cleavage, but the recent demonstration that a detergent-solubilized enzyme could accurately cleave pre-growth hormone and pre-prolactin (Jackson & Blobel, 1977) does not support this mechanism. More likely, similar secondary or tertiary structures of these proteins must be recognized by the enzyme although the membrane may facilitate cleavage since several pre-proteins are poor substrates for the solubilized enzyme.

If the cleavage of the pre-sequence of the precursor forms of secretory proteins is a single endoproteolytic cleavage, the intact cleaved pre-sequence should be present in the reactions. Pre-ProPTH is a particularly useful precursor for examining this question because 5 of the 7 methionines in the polypeptide are in the pre-sequence. In the studies reported here, we have been unable to detect any new peptides in the molecular weight range of this 25 amino acid sequence under conditions that easily detected the newly synthesized ProPTH. There is little apparent proteolytic activity in the wheat germ cell-free system as measured with substrates for chymotrypsin and trypsin (Roberts et al., 1974) and by the relative stability of the proteins synthesized in a standard 180-min reaction. It seems unlikely then that the pre-sequence is rapidly digested by wheat germ enzymes after it is cleaved from pre-ProPTH. While the fact that we cannot detect the pre-sequence is negative evidence and must be interpreted with caution, the experiments suggest that the removal of the pre-sequence might occur by some sequential exopeptidase mechanism or alternatively the pre-sequence might be rapidly and specifically degraded by enzymes associated with the stripped rough microsomes. These observations conflict with those of Jackson & Blobel (1977) who detected two small polypeptides that possibly corresponded to the pre-sequences of pre-growth hormone and pre-prolactin. Additional information gained from the studies of the cleavage of pre-ProPTH and other secretory protein precursors will be required to establish the mechanism of this proteolytic cleavage and explain its remarkably accurate cleavage of proteins with varied structures at the site of cleavage.

Acknowledgments

We acknowledge the contributions of Mr. John Coyne during the initial stages of this work. We thank Dr. John Zehr for providing dog pancreases and Calihan and Co., Peoria, Ill., for supplying bovine parathyroid glands.

References

Birken, S., Smith, D. L., Canfield, R. E., & Boime, I. (1977) *Biochem. Biophys. Res. Commun. 74,* 106–112.

Blobel, G., & Dobberstein, B. (1975) *J. Cell Biol. 67,* 852–862.

Blobel, G., & Sabatini, D. D. (1971) *Biomembranes 2,* 193–195.

Boime, I., Szczesna, E., & Smith, D. (1977) *Eur. J. Biochem. 73,* 515–520.

Burstein, Y., & Schechter, I. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 716–720.

Chu, L. L. H., MacGregor, R. R., Hamilton, J. W., & Cohn, D. V. (1974) *Endocrinology 95,* 1431–1438.

Cohn, D. V., MacGregor, R. R., Chu, L. L. H., Kimmel, J. R., & Hamilton, J. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 1521–1525.

Goltzman, D., Callahan, E. N., Tregear, G. W., & Potts, J. T., Jr. (1976) *Biochemistry 15,* 5076–5082.

Gray, W. R. (1967) *Methods Enzymol. 11,* 469–475.

Habener, J. F., Kemper, B., Potts, J. T., Jr., & Rich, A. (1975) *Biochem. Biophys. Res. Commun. 67,* 1114–1121.

Habener, J. F., Potts, J. T., Jr., & Rich, A. (1976) *J. Biol.*

Chem. 251, 3893-3899.
Habener, J. F., Amherdt, M., & Orci, L. (1977a) *Trans. Assoc. Am. Physicians 90,* 366-379.
Habener, J. F., Chang, H. T., & Potts, J. T., Jr. (1977b) *Biochemistry 16,* 3910-3917.
Habener, J. F., Rosenblatt, M., Kemper, B., Kronenberg, H. M., Rich, A., & Potts, J. T., Jr. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 2616-2620.
Hamilton, J. W., Niall, H. D., Jacobs, J. W., Keutmann, H. T., Potts, J. T., Jr., & Cohn, D. V. (1972) *Proc. Natl. Acad. Sci. U.S.A. 71,* 653-656.
Harrison, T. M., Adesnik, M., & Milstein, C. (1974) *Eur. J. Biochem. 47,* 613-620.
Hickey, E. D., Weber, L. H., & Baglioni, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 19-23.
Jackson, R. C., & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 5598-5602.
Jacobs, J. W., Kemper, B., Niall, H. D., Habener, J. F., & Potts, J. T., Jr. (1974) *Nature (London) 249,* 155-157.
Kemper, B. (1976) *Nature (London) 262,* 321-323.
Kemper, B., Habener, J. F., Potts, J. T., Jr., & Rich, A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 643-647.
Kemper, B., Habener, J. F., Mulligan, R. C., Potts, J. T., Jr., & Rich, A. (1974) *Proc. Natl. Acad Sci. U.S.A. 71,* 3731-3735.
Kemper, B., Habener, J. F., Potts, J. T., Jr., & Rich, A. (1976) *Biochemistry 15,* 15-20.
Lingappa, V. R., Devilliers-Thiery, A., & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2432-2436.
MacGregor, R. R., Chu, L. L. H., & Cohn, D. V. (1976) *J. Biol. Chem. 251,* 6711-6716.
McDowell, M. J., Joklik, W. K., Villa-Komaroff, L., & Lodish, H. F. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 2649-2653.
Milstein, C., Brownlee, G. G., Harrison, T. M., & Mathews, M. B. (1972) *Nature (London), New Biol. 239,* 117-120.
Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67,* 247-256.
Roberts, B. E., Paterson, B. M., & Sterling, R. (1974) *Virology 59,* 307-313.
Rosbash, M., & Penman, S. (1971) *J. Mol. Biol. 59,* 227-241.
Sabatini, D. D., Tashiro, Y., & Palade, G. E. (1966) *J. Mol. Biol. 19,* 503-524.
Shields, D., & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2059-2063.

κ Chain Variable Regions from Three Galactan Binding Myeloma Proteins†

D. Narayana Rao,‡ Stuart Rudikoff,* and Michael Potter

ABSTRACT: A series of seven BALB/c myeloma proteins has been identified with binding specificity for antigens containing β(1 → 6)-D-galactopyranosyl moieties. We have determined the primary amino acid sequence of the first 108 residues from the light chains of three of these proteins. The framework portions of the variable regions of these three light chains are identical with residue 100 at which position three different amino acids are found in the three chains. An additional interchange was found at position 106 in one of the proteins. Based on recent DNA sequence studies suggesting that the variable region ends at residue 97, these substitutions indicate the possible existence of multiple genes coding for the region beginning at residue 98 and continuing toward the carboxy terminus. A single amino acid interchange was observed in complementarity determining regions occurring in L3. This substitution (Ile-Trp) would require changes in all three codon bases to produce the respective amino acids if one were derived from the other. Two of these chains are thus indistinguishable for their first 100 amino acids and are the first pair of κ chains to exhibit complete identity over their variable regions.

One of the oldest and most intriguing problems in immunology is the question of the mechanism(s) involved in the generation of the vast array of antibody diversity. For the past several years attempts have been made to approach this problem by amino acid sequence analysis (Hood et al., 1970, 1976; Weigert et al., 1970; Rudikoff & Potter, 1976; Haber et al., 1976; Capra et al., 1976; Weigert & Riblet, 1976) and nucleic acid hybridization studies (Leder et al., 1976; Tonegawa et al., 1976). The availability of inbred mouse strains and the ability to induce myeloma proteins in certain of these strains (Potter, 1972) has greatly facilitated these experimental approaches. The use of this system has thus permitted an evaluation of protein structures derived from a genetically homogeneous population and avoids many of the problems introduced by genetic polymorphism in outbred populations.

The initial studies on mouse λl chains by Weigert et al. (1970) demonstrated that, in the case of λl where a total of 18 proteins have now been sequenced, 11 are identical throughout the variable region. The remaining seven differ by no more than three amino acids with all substitutions occurring in complementarity determining regions (CDR). This result led these investigators to suggest that a single germ line gene coded for λl chains and that the variants arose by somatic mutations which could then be clonally expanded by antigenic selection. This conclusion was extended to propose that the number of germ line genes was small and that diversity was generated through somatic mutation.

Results from studies on mouse κ chains reveal a much more complex system as evidenced by the large number of mouse κ isotypes or subgroups, each of which presumably requires a

† From the Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received July 10, 1978.*
‡ Visiting fellow, National Cancer Institute.