Porcine Proparathyroid Hormone. Identification, Biosynthesis, and Partial Amino Acid Sequence†

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ABSTRACT: Porcine parathyroid gland slices were incubated with ^{3}H-labeled amino acids in order to label tissue proteins. After incubation a crude hormonal extract was prepared and analyzed by chromatography on carboxymethylcellulose. Among the three radioactive peaks which were detected in the eluate, two were identified as parathyroid hormone and proparathyroid hormone. Based on thin layer gel filtration in the presence of 6 *M* guanidine-HCl, the proparathyroid hormone had a molecular weight of 11,500 compared to about 9600 for parathyroid hormone. Radioisotope sequence analysis of the proparathyroid hormone revealed a partial sequence of: Lys^1-Pro^2-Ile^3-Lys^4-Lys^5-Arg^6-Ser^7-Val^8-Ser^9--Ile^{11}--Met^{14}--Gly^{18}--Ser^{22}-Ser^{23}---. Thus, from position 7 onward the relative position of each amino acid tested in this molecule corresponded exactly to that in the porcine parathyroid hormone sequence. The conservation of a similar, though not identical, basic hexapeptide grouping Lys-X-Y-Lys-Lys-Arg- at the amino terminal region of the prohormone in all species examined thus far (porcine, human, and bovine) suggests that this segment of the molecule may play an important role in the conversion of the prohormone to the hormone.

Proparathyroid hormone (ProPTH)[1] has been discovered to be the biosynthetic precursor of parathyroid hormone in bovine (Hamilton et al., 1971a; Cohn et al., 1972; Kemper et al., 1972), human (Habener et al., 1972; Chu et al., 1973a), rat (Chu et al., 1973b), and chicken (MacGregor et al., 1973) parathyroid glands. In all of these species, the prohormone appears to consist of a single peptide chain. The partial amino acid sequences of the amino-terminal region of the bovine and human prohormones have been reported recently (Hamilton et al., 1974; Jacobs et al., 1974; Huang et al., 1975). Both molecules start at the amino-terminus with the identical hexapeptide sequence Lys-Ser-Val-Lys-Lys-Arg- which is followed by the sequence of the respective PTH molecule. Evidence exists also that the b- and hProPTH's both contain an additional peptide subsequent to the carboxy-terminus of the hormone sequence (Huang et al., 1975; Hamilton et al., 1975). It is visualized that conversion of ProPTH to PTH entails specific cleavages of these amino- and carboxy-terminal peptides (Hamilton et al., 1975).

Recently the complete amino acid sequence for porcine PTH was reported (Sauer et al., 1974). Since ProPTH has been found in the parathyroid glands of all animal species examined thus far, it seemed likely that pProPTH would also exist. If ProPTH exists, one might naturally ask whether its general structure relative to PTH is the same as those two already described. More specifically, does it contain the same amino-terminal hexapeptide as do b- and hProPTH? Answers to these questions should be of aid to our understanding of the mechanisms of the biological conversion of ProPTH to PTH. For this reason, we initiated a study on the biosynthesis of PTH in the porcine parathyroid gland.

This report describes the identification and biosynthesis of both ProPTH and PTH by porcine parathyroid gland slices; provides data on the partial amino acid sequence of the porcine prohormone; and compares the porcine prohormone to its bovine and human counterparts.

Experimental Section

Methods

Isolation of Radioactive Peptides. Incubation of fresh porcine parathyroid glands with ^{3}H-labeled amino acid was performed as described earlier for human parathyroid gland (Chu et al., 1973a). In some studies which were designed to determine the amino acid sequence of pProPTH and pPTH, combinations of two amino acids—one ^{3}H-labeled and one ^{14}C-labeled—were used instead of a single ^{3}H-labeled amino acid. After incubation, the mixture was frozen quickly until it was processed.

To each thawed sample of incubated porcine parathyroid tissue and incubation medium was added 3 g of nonradioactive bovine or porcine parathyroid tissue and the mixture was processed as described earlier for the isolation of the rat and human PTH and ProPTH (Chu et al., 1973a,b). In this procedure the tissue is homogenized in the urea–HCl–cysteine solution and subjected to organic solvent, salt, and trichloroacetic acid precipitation to yield a Cl_3CCOOH powder. This is then subjected to ion-exchange chromatography on carboxymethylcellulose in order to separate ProPTH and PTH from each other and from other labeled proteins.

Migration Behavior of pProPTH and pPTH on Sephadex Gels. Thin layer gel filtration (Sephadex G-100, super-

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[1] Abbreviations used are: PTH, parathyroid hormone; ProPTH, proparathyroid hormone; b-, h-, and p- preceding PTH or ProPTH refer to the bovine, human, and porcine molecules, respectively; I-PTH, immunoreactive PTH; Cl_3CCOOH powder, trichloroacetic acid powder (a crude hormonal preparation from parathyroid glands); CM-cellulose, carboxymethylcellulose.

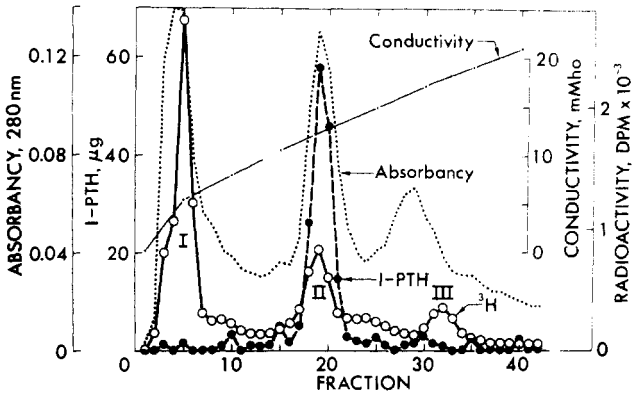

FIGURE 1: CM-cellulose chromatography of porcine parathyroid Cl_3CCOOH powder. The Cl_3CCOOH powder was prepared from a mixture of 40 mg of porcine parathyroid tissue which was incubated for 2 hr and 3 g of carrier porcine tissue. The sample was applied to a CM-cellulose column (2.5 × 100 mm) and eluted with a linear ammonium acetate gradient from 0.01 *M*, pH 5.3 to 0.30 *M*, pH 7.0 containing 1 m*M* mercaptoethanol. Fractions of 1.5 ml were collected.

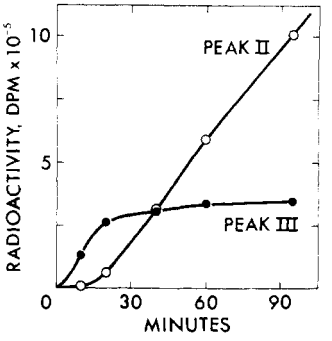

FIGURE 2: Time-course of incorporation of [^{3}H]leucine into radioactive peak II and radioactive peak III. Batches of 40 mg of porcine parathyroid tissue were incubated with [^{3}H]leucine for the indicated period of time, after which the tissues were processed together with carrier nonradioactive bovine tissue. Radioactive peaks II and III were isolated by CM-cellulose column chromatography as shown in Figure 1.

fine) was performed according to the procedure employed by Cohn et al. (1974a). Positions of the peptides on the paper replica of the thin layer plate were detected either by fluorescamine or by assaying radioactivity on the paper after cutting the paper strip into 3-mm segments.

Column gel filtration was performed with Sephadex G-100 (superfine) in a 2.5 × 45 cm column as described earlier (Hamilton et al., 1971a). Relative migration (R_f) is defined as sample volume (V_s) divided by void volume (V_0).

Radioisotope Microanalysis of Peptide Sequence. The procedure was that of Huang et al. (1975). Edman degradation of the sample together with apomyoglobin as carrier was performed with a Beckman Sequencer, Model 890C, employing a Slow Protein-Quadrol program (Beckman Sequencer Manual, 1971). The resultant thiazolinones which contained elevated radioactivity were converted to the phenylthiohydantoin amino acid derivatives and were analyzed together with authentic derivatized amino acids by ascending chromatography on silica gel thin plates.

Immunoassay. The immunoreactivity of samples was assayed by radioimmunoassay as described earlier (Hamilton et al., 1971b) using guinea pig antiserum to bPTH (antiserum GP-6 at a final dilution of 1:80,000). Bovine PTH was iodinated with ^{125}I by the method of Hunter and Greenwood (1962). Homogeneous bPTH prepared in our laboratory (Hamilton et al., 1971b) was used to construct the standard curve. The porcine Cl_3CCOOH powder yielded an assay curve which was superimposable on the curve obtained with bPTH standard.

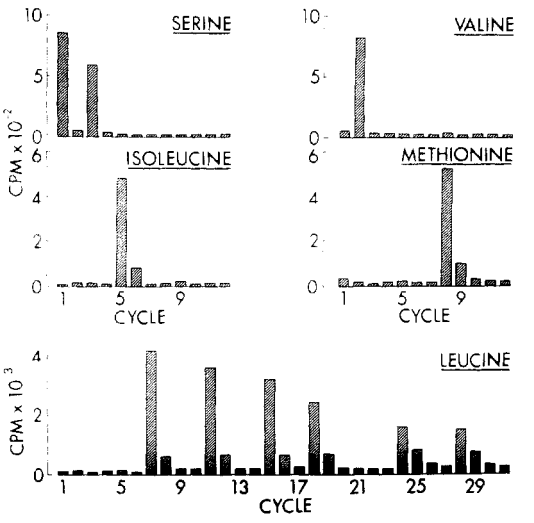

FIGURE 3: Automated Edman degradation of radioactive peak II. [^{3}H]Serine, [^{3}H]valine, [^{3}H]isoleucine, [^{3}H]methionine, or [^{3}H]leucine labeled peak II (about 8 × 10^3, 9 × 10^3, 2.5 × 10^3, 4 × 10^3, and 6 × 10^3 cpm, respectively) were subjected to the indicated numbers of cycles of Edman degradation. Aliquots of the thiazolinone derivative at each cycle were assayed for radioactivity.

Materials

Radioactive amino acids were purchased from New England Nuclear (Boston, Mass.), ^{125}I for radioimmunoassay was from Industrial Nuclear Co. (St. Louis, Mo.), CM-cellulose (CM-52) was from Reeve Angel (Clifton, N.J.), reagents for the Edman degradation were from Beckman Instruments, Inc. (Palo Alto, Calif.) and Pierce Chemical Co. (Rockford, Ill.), and apomyoglobin was from Beckman Instruments. Bovine PTH and ProPTH labeled with [^{14}C]leucine were prepared in our laboratory (Cohn et al., 1972).

Results

Figure 1 shows CM-cellulose chromatographic profiles of absorbancy, radioactivity, and immunoreactivity in a Cl_3CCOOH powder prepared from a mixture of radioactive and carrier porcine parathyroid tissue. The radioactive peptides were resolved into three major peaks centering at fraction 5 (radioactive peak I), fraction 19 (radioactive peak II), and fraction 32 (radioactive peak III). This profile of radioactivity resembled that obtained when radioactive bovine parathyroid tissue was examined (Chu et al., 1974). A major immunoreactive peak (I-PTH) and a major peak of absorbancy both coeluted with radioactive peak II. No immunoreactivity was detected in radioactive peaks I and III. These results indicated that radioactive peak II likely consisted of pPTH and, in accord with our experience in the study of bovine, rat, and human ProPTHs, radioactive peak III represented pProPTH.

When radioactive porcine parathyroid glands were processed together with carrier bovine tissue, a similar profile of radioactivity and absorbancy was obtained. In this case, however, the radioactive peak II eluted slightly later than the bPTH protein peak. This result was expected since pPTH is more basic than its bovine counterpart (Sauer et al., 1974).

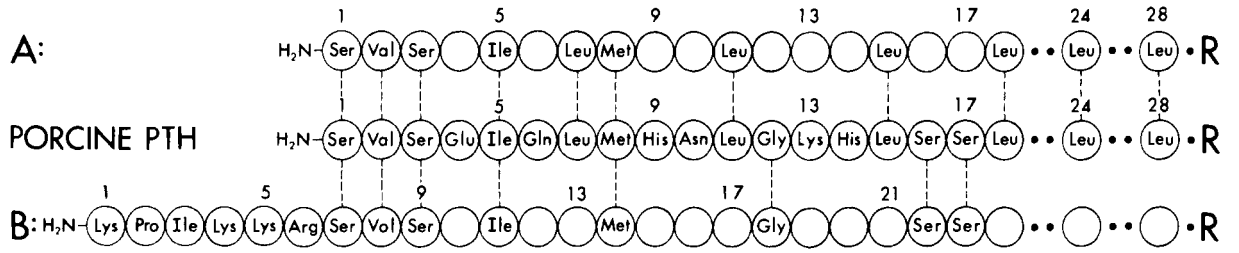

FIGURE 4: Summary of radioisotope microsequencing results. (A) Schematic presentation of results obtained with radioactive peak II (see Figure 3). (B) Schematic presentation of results obtained with radioactive peak III (see Figure 5). Part of the established amino acid sequence of pPTH (Sauer et al., 1974) is presented in the center of the figure.

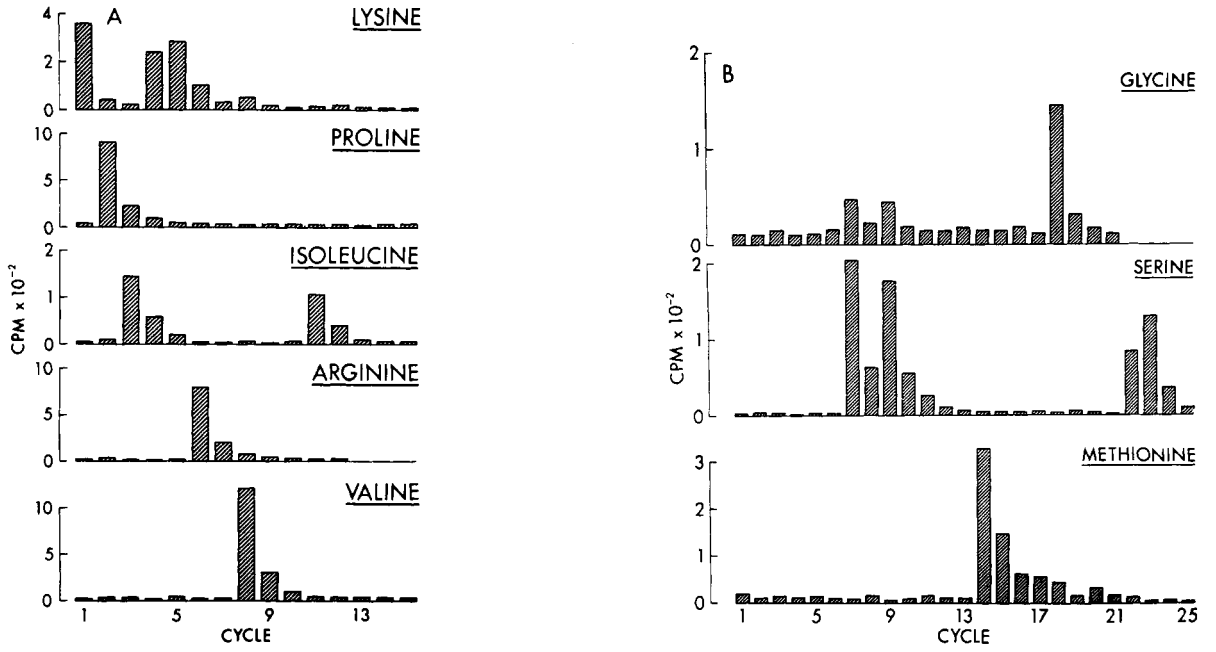

FIGURE 5: Automated Edman degradation of radioactive peak III. (A) [^{3}H]Lysine-, [^{3}H]proline-, [^{3}H]isoleucine-, [^{3}H]arginine-, [^{3}H]valine-labeled peak III (about 7×10^3, 4×10^3, 1×10^3, 9×10^3, and 18×10^3 cpm, respectively) were subjected to 12 cycles of Edman degradation. (B) [^{3}H]Glycine-, [^{3}H]serine-, and [^{3}H]methionine-labeled peak III (3×10^3, 5×10^2, and 13×10^3 cpm, respectively) were subjected to up to 24 cycles of Edman degradation. Aliquots of the thiazolinone derivative at each cycle were assayed for radioactivity.

Figure 2 portrays a time course of incorporation of [^{3}H]leucine into peaks II and III. Radioactive peak III formed immediately and plateaued after 20 min. Radioactive peak II appeared after 10 min and thereafter increased steadily during the course of incubation. Thus peak III and peak II—the putative pProPTH and pPTH—exhibited the apparent precursor-product relationship resembling those previously described for ProPTH and PTH in bovine, rat, and human parathyroid glands (Cohn et al., 1972; Chu et al., 1973a,b).

When radioactive peaks II and III were subjected to thin layer gel filtration in the presence of 6 *M* guanidine hydrochloride, each species migrated as a single symmetrical spot. Their respective molecular weights were estimated to be about 11,500 and 9600.

Definitive evidence that radioactive peak II was pPTH was obtained by radioisotope microsequencing. The results of a series of Edman degradations are shown in Figure 3. The sample labeled with [^{3}H]serine yielded elevated radioactivity at cycles 1 and 3; with [^{3}H]valine, at cycle 2; with [^{3}H]isoleucine, at cycle 5; with [^{3}H]methionine, at cycle 8; and with [^{3}H]leucine, at cycles 7, 11, 15, 18, 24, and 28. At each of these cycles the phenylthiohydantoin derivative was found to comigrate with the authentic derivatized amino acid upon thin layer silica gel. These results show that the amino acid sequence of peak II was Ser^1-Val^2-Ser^3--Ile^5--Leu^7-Met^8--Leu^{11}---Leu^{15}--Leu^{18}---Leu^{24}---Leu^{28}--- (Figure 4A), which corresponds to that of pPTH (Figure 4, center).

When batches of radioactive peak III were subjected to similar analysis the sample labeled with [^{3}H]lysine yielded elevated radioactivity at cycles 1, 4, and 5; with [^{3}H]proline, at cycle 2; with [^{3}H]isoleucine, at cycles 3 and 11; with [^{3}H]arginine, at cycle 6; with [^{3}H]valine, at cycle 8; with [^{3}H]glycine, at cycle 18; with [^{3}H]serine, at cycles 7, 9, 22, and 23; and with [^{3}H]methionine, at cycle 14 (Figure 5). In one study in which radioactive peak III was labeled with [^{3}H]proline and [^{14}C]glycine, the sample yielded elevated ^{3}H radioactivity at cycle 2 and ^{14}C radioactivity at cycle 18. These results indicated that the amino acid sequence of peak III is Lys^1-Pro^2-Ile^3-Lys^4-Lys^5-Arg^6-Ser^7-Val^8-Ser^9--Ile^{11}-Met^{14}---Gly^{18}---Ser^{22}-Ser^{23}--- (Figure 4B). It is apparent that peak III differs in part from pPTH by having the hexapeptide Lys-Pro-Ile-Lys-Lys-Arg- at the amino-terminal region of pPTH. These data establish that peak III represents porcine proparathyroid hormone.

When ^{3}H-labeled pPTH was cochromatographed with ^{14}C-labeled bPTH on a Sephadex G-100 gel column, the porcine hormone eluted slightly earlier than did the bovine hormone (R_f: bPTH = 1.80; pPTH = 1.75). Similarly, ^{3}H-

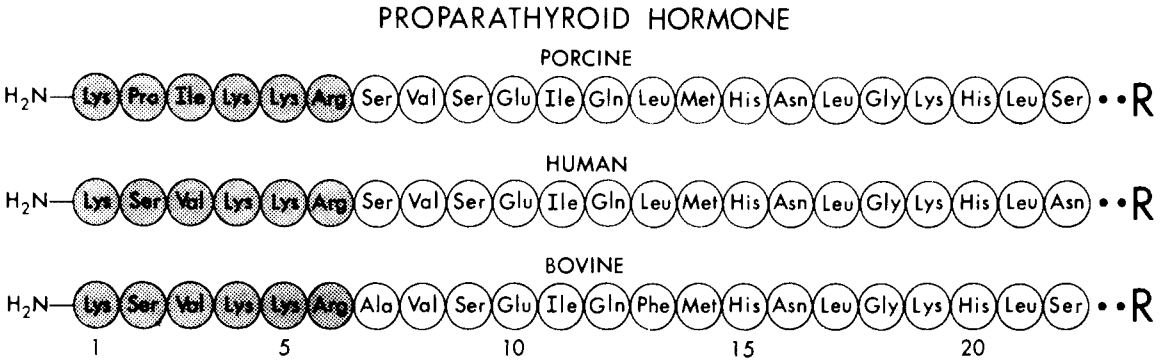

FIGURE 6: Partial amino acid sequences of porcine, human, and bovine proparathyroid hormone. The unshaded amino acid residues represent the PTH sequences of the respective species.

labeled pProPTH eluted slightly earlier than did ^{14}C-labeled bProPTH (R_f: bProPTH = 1.70; pProPTH = 1.66). These results indicate that the hydrodynamic radii of both pPTH and pProPTH are greater than the respective bovine molecules. When ^{3}H-labeled pProPTH was cochromatographed on CM-cellulose with ^{14}C-labeled bProPTH, the porcine molecule eluted after its bovine counterpart (buffer conductivity for pProPTH, 16.5 mmho; for bProPTH, 15.0 mmho). This result suggests that the pProPTH is more basic in ionic character than bProPTH as is also the case with pPTH compared to bPTH.

Discussion

This report demonstrates that from porcine parathyroid tissue a radioactive peptide can be isolated consisting of the pPTH sequence with an additional hexapeptide at the amino terminus comparable to similar peptides in human and bovine species. The results of the sequence analysis, its size, and the kinetics relationship to pPTH all support the conclusion that this peptide is a prohormone of pPTH. Thus the present report provides yet additional evidence that the pathway proPTH to PTH is a general one in higher animals.

The partial amino acid sequence of pProPTH as determined in the present study together with those of hProPTH and bProPTH (Hamilton et al., 1974; Jacobs et al., 1974; Huang et al., 1975) are provided in Figure 6. Because the amino-terminal hexapeptide sequences of the bovine and human prohormones were found to be identical, we originally speculated that this portion of all of the ProPTH's might be similar. The present report reinforces this notion, since the amino-terminal hexapeptide of the porcine molecule is similar, although not identical, to that in the bovine and human prohormones. Two of the residues in this hexapeptide portion of pProPTH differ from those in the bovine and human molecules: proline is substituted for serine in position 2, and isoleucine for valine in position 3. These substitutions are not unique in nature (Dayhoff, 1972).

In the ProPTH's analyzed thus far these variations in amino acid do not alter the unique basicity of the hexapeptide since the sequence Lys-X-Y-Lys-Lys-Arg- is present in all. This conservation of grouping of these basic amino acids may be more than fortuitous and strengthens the speculation (Huang et al., 1975) that the amino-terminal hexapeptide of ProPTH may have an important physiological role in its biosynthesis, in its transfer through the cisternal space of the smooth endoplasmic reticulum to the Golgi zone or in the process of its conversion to the hormone (Cohn et al., 1974b, 1975). For example, the identical Lys-Lys-Arg- sequence which directly precedes residue 1 of the respective PTH sequences points up the likely existence in the parathyroid cells of various species of similar, if not identical, proteolytic enzyme systems for the removal of the hexapeptide from the prohormone during the process of its conversion to the hormone.

Based in part upon amino acid composition, estimation of molecular weight and preliminary analysis of the carboxy-terminal region of bProPTH, we have suggested that an additional sequence of up to 15 amino acids might follow the carboxy-terminal residue of bPTH (Hamilton et al., 1975). Since pProPTH appears to have a molecular weight about 1900 greater than that of pPTH, and since the amino-terminal hexapeptide can account for only about 800, it is likely that the porcine prohormone also has a substantial carboxy-terminal adduct attached to the carboxy-terminal residue of the porcine such as appears to be the case for hProPTH (Huang et al., 1975).

Judged from their migration behavior during gel filtration chromatography, we showed previously that bProPTH and bPTH contain some structure which is highly asymmetrical (Cohn et al., 1974a). That finding together with migrations of pProPTH and pPTH measured in the present study and of hProPTH and hPTH reported earlier (Chu et al., 1973a) indicate that a highly asymmetrical tertiary structure is a feature shared by the prohormone and hormone molecule of these, and possibly all, species. The physiological significance of such physical and chemical properties must yet be established.

Finally, we should note that the sequence data reported in the present study were made possible through the use of the highly sensitive radioisotope sequencing methodology. With this approach we were able to obtain the present results with a total of less than 10 μg of ProPTH. This should be compared to the near milligram amounts required for a standard sequencing procedure (Hamilton et al., 1974). It is expected that radioisotope microsequencing will be generally applicable to proteins which can be biosynthesized by fresh tissue, organ culture, microorganisms, or cell free systems (Tager et al., 1973; Schachter, 1973) and will be particularly useful in studying the primary structures of peptides and proteins which are available in only limited quantities.

Acknowledgment

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Controlled Digestion with Trypsin as a Structural Probe for the N-Terminal Peptide of Soluble and Membranous Cytochrome c_1†

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ABSTRACT: When purified bovine cytochrome c_1 is digested with trypsin under controlled conditions, the heme polypeptide is preferentially converted from a species of molecular weight 30,600 to a heme polypeptide of molecular weight 29,000. The trypsin sensitive peptide bond is located in the N-terminal region of the cytochrome. Both the reduced and oxidized cytochrome are susceptible to hydrolysis by trypsin at the same locus, but the reduced cytochrome is cleaved at an initial rate approximately twofold greater than the oxidized cytochrome. Membranous cytochrome c_1, as occurring in cytochrome b-c_1 complex or succinate-cytochrome c reductase complex, is not susceptible to trypsin proteolysis under similar conditions, nor after more extensive treatment of the membranes with trypsin, in spite of the fact that cytochrome c_1 presumably comes into contact with cytochrome c at the membrane surface during electron transport. These findings are consistent with a model for the structure of cytochrome c_1 in situ in which the cytochrome is an integral membrane protein, located primarily in the membrane continuum, while still having the heme-containing portion of the protein available at the membrane surface for electron transfer to cytochrome c.

The proteins which make up the electron transport chain appear to fulfill both a structural and a functional role in the inner mitochondrial membrane. Although there have been numerous investigations of the relationship between structure and function of these components of the inner mitochondrial membrane, there has been no precedent attempt to elucidate the three-dimensional structure of a single mitochondrial membrane protein in situ.

Cytochrome c_1 is particularly suitable as a subject for investigation of protein structure within the mitochondrial membrane. In addition to its obligatory function in electron transport, cytochrome c_1 is a membrane protein of a type which cannot be dissociated from the inner membrane without concomitant destruction of the membrane structure and loss of associated membrane activities. It thus differs from such mitochondrial proteins as cytochrome c and the AT-

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