Amino Acid Sequences of Tryptic Peptides of Cytochromes $b_5$ from Microsomes of Human, Monkey, Porcine, and Chicken Liver*

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SUMMARY

Liver microsomal apocytochromes $b_5$ from man, monkey (Alouatta fusca), pig, and chicken were subjected to trypsin digestion, and all of the peptides were isolated and characterized. The sum of the residues present in these peptides equaled the total amino acid composition of the corresponding parent cytochrome. For all of these peptides a homologous segment was found in our previously established bovine and rabbit cytochrome sequence. This information provided sufficient evidence to construct a unique amino acid sequence for human, monkey, porcine, and chicken cytochrome $b_5$. Comparison of the six sequences indicated a close similarity between these proteins. The human cytochrome differed from that of chicken in 15 positions but from that of monkey in only 2 positions. With the exception of the avian protein sequence, the amino acid replacements were confined predominantly to the NH$_2$- and COOH-terminal segments of the cytochrome. A segment comprising residues 42 to 72 was invariant in all six cytochromes.

One intriguing aspect of heme protein structures is that they represent a system in which particular amino acid arrangements of the peptide chain can induce the heme to participate in diverse catalytic functions. Cytochrome $b_5$ is a heme protein present in especially high amount in the endoplasmic reticulum of mammalian liver cells (1). Moreover, Halloway and Wakil (2) have shown that cytochrome $b_5$ is an integral part of the stearic coenzyme A desaturase system.

Information about the function and, in particular, the primary structure of heme proteins has been accumulating at a remarkable rate during the past few years. In the last decade, the amino acid sequences of cytochromes $c$ from about 36 different organisms have been determined, and complete or partial primary structures of hemoglobins of some 20 species are now known (3). The implications derived from these sequences have firmly established the significance of the sequence data in the elucidation of structure and evolution of these proteins. Liver microsomal cytochrome $b_5$ has properties that resemble those of the cytochromes $c$, and also those of the hemoglobin. Thus, in several aspects, cytochrome $b_5$ is a member of both the cytochrome $c$ and the hemoglobin group. The heme structure and ligand-binding properties of cytochrome $b_5$ parallel those of the hemoglobins (4). The enzymic and spectral properties of cytochrome $b_5$, however, are strongly related to those of the cytochrome $c$. An exclusive property of cytochrome $b_5$ is its strong association in vivo with the cellular membrane components (1).

In previous studies, we determined the covalent structures of calf (5-7) and rabbit (8) liver microsomal cytochromes $b_5$ and briefly discussed the unexpected sequence similarity between this cytochrome and hemoglobin (1). It was also observed that in the rabbit protein heterogeneity at residues 10 and 95 was present (8). In order to explain these findings and to elucidate further the phylogenetic and the structural aspects of this group of proteins, we determined the amino acid sequences of cytochrome $b_5$ from man, monkey, pig, and chicken. Independent studies by Tsugita et al. (9) have also established the rabbit cytochrome $b_5$ sequence. For the first 90 residues, these amino acid sequences are essentially the same.

EXPERIMENTAL PROCEDURE

Materials and Methods—Microsomal liver cytochromes were obtained as previously described by Nobrega et al. (10), and, following lyophilization, were stored in glass ampoules in vacuo. The purity of the material was ascertained by the following criteria: the preparations migrated as a single band on DEAE-Sephadex A-50 columns, and when electrophoresed on the cellulose acetate strips in phosphate or acetate buffers (11). The cytochromes used had an absorbance ratio, $A_{550}$ reduced to $A_{550}$ oxidized of 1.4 or higher and were homogeneous by end group analysis using the dansyl chloride method (12).

* The abbreviations used are: dansyl-, 1-dimethylaminonaphthalene-5-sulfonyl, PTH-, phenylthiohydantoin derivative; ND, not determined.
Enzymic Hydrolysis and Isolation of Peptides—Heme-free apocytochrome (0.1 to 0.5 μmole) was prepared by treatment of the cytochrome with 25% acetic acid at 0°C as follows: to a 0.5-ml solution of salt-free cytochrome in 10 ml of cold acetone containing 0.2% HCl (v/v) were added. After 10 min at 5°C, white precipitated apocytochrome was collected by centrifugation, rapidly dried with a stream of nitrogen, and dissolved in a total volume of 0.5 ml of 0.1 M NH₄HCO₃, pH 8.1. A 0.1% solution of trypsin ( Worthington, 1-tosylamido-2-phenyl-ethyl chloromethyl ketone-treated) in 0.001 M HCl was prepared immediately prior to use and added to the apoprotein to give an enzyme to protein ratio of 1:60. After 3 hours at 25°C, the hydrolysate was lyophilized and dissolved in the buffer appropriate to the desired chromatographic procedure. The chicken apoprotein was dissolved in 0.1 M Tris-8 M urea buffer, pH 8.1, and the protein solution was diluted 3 times before trypsin addition to reduce the urea concentration to 2 M. After 1 hour at 25°C, another aliquot of trypsin was added, and the hydrolysate was allowed to proceed for another 5 hours. Peptides were digested with chymotrypsin ( Worthington, crystallized three times) in 0.1 M ammonium bicarbonate, pH 8.1, at 25°C for 3 hours, with an enzyme to substrate ratio of 1:100. Porcine Peptide T-1 was digested with pepsin (Worthington, crystallized three times), 0.2 μmole of peptide, 2% (moles per mole) enzyme, 1.5 ml of 0.01 M HCl, 25°C, 13 hours.

The chromatographic systems used were Dowex 1 (AG-1W-X2, 200 to 400 mesh, Bio-Rad) and Dowex 50 (AG-50W-X2, 200 to 400 mesh, Bio-Rad) columns, 0.9 × 60 cm, at 40°C at a flow rate of 20 ml per hour, using morpholine- , picoline- , pyridine-acetic acid buffers as previously described. Fractions of 1.5 ml were collected.

Peptide fractionations by gel filtrations were performed on columns, 0.9 × 60 cm, of Sephadex G-25, equilibrated and eluted with 30% acetic acid.

Peptide fractions were identified by the ninhydrin reaction after alkaline hydrolysis and characterized by amino acid analysis. Tryptic peptides are assigned the letter T and the peptic peptides the letter P. The number assigned to these residues in these sequences corresponds to the number of the homologous residue of the rabbit protein sequence (5).

Amino Acid Analyses of Proteins and Peptides—Amino acid compositions were determined on acid hydrolyses with the Spinco model 120C amino acid analyzer, equipped with a 6.6-mm flow cuvette and a 4- to 5-mv range recorder, permitting the determination of amino acids in the range of 0.0025 to 0.03 μmole. Calculation of the number of residues per molecule of protein was based on contents of one heme group per molecule of protein. Peptides were hydrolyzed routinely in 6 N HCl at 107°C in evacuated tubes for 22 hours. A duplicate sample was hydrolyzed for 72 hours for peptides containing valine, isoleucine, or leucine. Tryptophan was determined on the amino acid analyzer from a 20-hour hydrolysate containing 3% (v/v) thioglycolic acid (mercaptoacetic acid, Eastman) (13). Following this hydrolysis the samples were taken to dryness overnight in a vacuum desiccator containing pellets of KOH, and then lyophilized for 1 hour with a high diffusion capacity vacuum pump. Since hydrolysates containing thioglycolic acid have a number of extra peaks on the amino acid analyzer (near the arginine, aspartic acid, and valine elution positions), appropriate blanks were run. For an improved recovery (95% +) of tyrosine from acid hydrolysates a 1% (v/v) thioglycolic acid concentration was used.

Methods of Sequence Analysis—The sequential degradation of Edman (14) were performed. The coupling with phenylisothiocyanate and its cyclization in anhydrous trifluoroacetic acid were carried out under nitrogen. The identity of the NH₂-terminal residue was determined by amino acid analysis on a fraction of the residual peptide. The number of micromoles of amino acid present in the residual peptide was converted to a molar ratio, by dividing the analytical value of the residue by the mean value of the total composition. The mean value of the composition was obtained by dividing the sum of the analytical values of all amino acids by the assumed number of residues in the peptide. The assumed number of residues, if not evident, is given in parentheses following the molar ratio values. Amino acids present in peptides, before Edman degradations, in yields below 15% of a residue are not reported or included in the calculations. The residue marked in boldface type corresponds to the residue removed at each step. A 0.0 molar ratio, following an Edman degradation step, represents less than 0.1 residue. When Edman degradation results are reported in a column form, the column heading C represents the initial peptide composition, and E- followed by a number denotes the composition after the corresponding degradation step. Direct identification and differentiation of the phenylthiohydantoins of glutamic and aspartic acids and their amides was carried out by thin layer chromatography, with solvent C as recommended by Edman and Sjoquist (15). Eastman precoated silica gel sheets were used without fluorescent indicator, and spots were located by spraying with 0.1 N iodine, 5% (v/v) sodium azide solution. Conversion of the phenylthiohydantoin to free amino acid was performed by acid hydrolysis with 6 N HCl at 150°C, for 16 hours.

The NH₂-terminal residues of the proteins were determined after reaction with dansyl chloride according to the method of Gray and Hartley (12). The derivatives were identified by two-dimensional thin layer chromatography and polyamide layers by solvent 1 (200 ml of water and 3 ml of 90% formic acid) and solvent 3 (60 ml of n-heptane, 60 ml of n-butyl alcohol, and 20 ml of glacial acetic acid) of Woods and Wang (16).

RESULTS

Amino Acid Composition and Terminal Residues of Cytochromes—The cytochromes investigated represent the predominant family obtained from the final purification procedure by chromatography on DEAE-Sephadex A-50 (11). These fractions were homogeneous as indicated by electrophoresis and spectral ratios of oxidized and reduced states. The composition of the cytochromes are listed in Tables I through IV. The number of residues of each amino acid in the protein is calculated on the basis of 1 mole of heme per mole of protein.

The human and the monkey proteins each contains a total of 87 residues. The porcine protein contains 82 residues, and the number assigned to a residue in these sequences corresponds to the residue removed at each step. A 0.0 molar ratio, following an Edman degradation step, represents less than 0.1 residue. When Edman degradation results are reported in a column form, the column heading C represents the initial peptide composition, and E- followed by a number denotes the composition after the corresponding degradation step. Direct identification and differentiation of the phenylthiohydantoins of glutamic and aspartic acids and their amides was carried out by thin layer chromatography, with solvent C as recommended by Edman and Sjoquist (15). Eastman precoated silica gel sheets were used without fluorescent indicator, and spots were located by spraying with 0.1 N iodine, 5% (v/v) sodium azide solution. Conversion of the phenylthiohydantoin to free amino acid was performed by acid hydrolysis with 6 N HCl at 150°C, for 16 hours.

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avian cytochrome has 83 residues. Dansylation procedure revealed serine as the NH₂-terminal residue in human and monkey cytochrome preparations. The same method revealed alanine as the NH₂-terminal residue of the porcine cytochrome and glycine as the NH₂-terminus of the chicken protein. These results are in complete agreement with the previous analyses made by Nobrega et al. (10).

Isolation and Characterization of Tryptic Peptides of Human Cytochrome bs—Peptides produced by tryptic digestion of human apocytochrome were separated on a Dowex 1 column giving the elution profile shown in Fig. 1C. Peptides T-3 and T-6 were not separated by this procedure but were resolved by gel filtration on Sephadex G-25 in 30% acetic acid. Peptide T-12 is insoluble in the basic morpholine-pipoline buffer. Gel filtration of this precipitate on Sephadex G-25 in 30% acetic acid or chromatography on a Dowex 50 column recovered Peptide T-12 in good yield. The amino acid analyses of the tryptic peptides of human cytochrome bs are given in Table I. The sum of the compositions of tryptic peptides agrees well with the analyses of the parent cytochrome. The yields represent the actual amount of the peptide with the reported composition obtained.

The two remaining residues were positioned as Val-Lys from our previously determined calf and rabbit protein sequences having composition and properties unambiguously identical with those of calf and rabbit proteins are listed without proof.

Table I

Amino acid composition of tryptic peptides of human apocytochrome bs

| Amino Acid | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | T-10 | T-12 | Total |
|-----------|-----|-----|-----|-----|-----|-----|-----|------|------|-------|
| Lysine    | 1.03| 0.85| 0.97| 1.07| 1.15| 1.23| 0.93| 0.95  | 1.17  | 7     |
| Histidine | 1.00| 0.97| 1.00| 1.00| 1.00| 1.00| 1.00| 1.00  | 1.00  | 10    |
| Arginine  | 0.97| 0.97| 1.00| 1.00| 0.94| 1.17| 1.17| 1.04  | 1.04  | 5     |
| Aspartic Acid | 0.92| 0.92| 1.00| 1.00| 1.00| 1.00| 1.00| 1.00  | 1.00  | 5     |
| Threonine | 1.97| 1.97| 1.97| 1.97| 1.97| 1.97| 1.97| 1.97  | 1.97  | 3     |
| Serine    | 1.02| 0.86| 0.96| 0.96| 0.96| 0.96| 0.96| 0.96  | 0.96  | 6     |
| Glutamic Acid | 4.32| 4.24| 0.89| 0.89| 0.89| 0.89| 0.89| 0.89  | 0.89  | 6     |
| Proline   | 0.86| 1.00| 0.70| 0.70| 0.70| 0.70| 0.70| 0.70  | 0.70  | 6     |
| Glycine   | 0.10| 0.10| 0.10| 0.10| 0.10| 0.10| 0.10| 0.10  | 0.10  | 6     |
| Alanine   | 3.10| 3.10| 3.10| 3.10| 3.10| 3.10| 3.10| 3.10  | 3.10  | 6     |

Values represent the mean result of 20- and 72-hour hydrolysates, calculated on the basis of 1 mole of heme per mole of protein. Serine and threonine values are extrapolated to zero hydrolysis time.

a Sample hydrolyzed in the presence of 1% thioglycolic acid.

b Sample hydrolyzed in the presence of 3% thioglycolic acid.

c Represent the actual amount of the peptide with the above composition obtained.

d Value from 72-hour hydrolysate.

TABLE I

| No. of Residues | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | T-10 | T-12 | Total |
|-----------------|-----|-----|-----|-----|-----|-----|-----|------|------|-------|
| Purification Procedure | DX1 | DX1 | DX1 | DX1 | DX1 | DX1 | DX1 | DX1 | DX50 |       |
| Yield (%)       | 69  | 55  | 51  | 57  | 19  | 21  | 50  | 53   | 50   |       |

The results are expressed as molar ratios of the constituent amino acids and, except where noted, were obtained by analysis of 20-hour hydrolysates. No corrections for the destruction of serine and threonine during hydrolysis are incorporated.

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The results were as follows: Step 1 (100%) : Asp, 4.09; Thr, 1.83; Ser, 0.50; Glu, 3.13; Gly, 2.00; Ala, 2.35; Val, 0.98; Phe, 1.07; Lys, His, and Arg, ND. Step 2 (95%) : Asp, 3.89; Thr, 1.69; Ser, 0.80; Glu, 3.84; Gly, 2.69; Ala, 2.76; Val, 1.03; Phe, 0.10; His, Lys, and Arg, ND. The phenylthiohydantoins from these steps were identified as PTH-glutamic acid (Step 1) and PTH-glutamine (Step 2).

Pep tide T-4 (Residues 52 through 72) : Glu-Gln-(Ala, Gly, Gly, -Asp, Ala, Thr, Glu, Asp, Phe, Glu, Asp, Val, Gly, His, Ser, Thr, -Asp, Ala) - Arg—This peptide was assumed to be homologous to Peptide T-1 of rabbit cytochrome b5 because their compositions and chromatographic behavior are identical. Two steps of the Edman degradation confirmed the expected Glu-Gln sequence. The results were as follows: Step 1 (100%) : Asp, 4.09; Thr, 1.83; Ser, 0.50; Glu, 3.13; Gly, 2.00; Ala, 2.35; Val, 0.98; Phe, 1.07; Lys, His, and Arg, ND. Step 2 (95%) : Asp, 3.89; Thr, 1.69; Ser, 0.80; Glu, 3.84; Gly, 2.69; Ala, 2.76; Val, 1.03; Phe, 0.10; His, Lys, and Arg, ND. The phenylthiohydantoins from these steps were identified as PTH-glutamic acid (Step 1) and PTH-glutamine (Step 2).

Pep tide T-5 (Residues 73 through 88) : His-Asn-His-Ser-Lys—Three steps of the Edman degradation revealed the NH2-terminal sequence as His-Asn-His.

The seryl-lysyl sequence was deduced from tryptic specificity. The serly-lysyl sequence was deduced from tryptic specificity. The serly-lysyl sequence was deduced from tryptic specificity.
The results are expressed as molar ratios of the constituent amino acids and, except where noted, were obtained by analysis of 20-hour hydrolysates. No corrections for the destruction of serine and threonine during hydrolysis are incorporated.

| Amino Acid | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | T-8 | T-12 | Total | Protein/
|------------|-----|-----|-----|-----|-----|-----|-----|-----|------|-------|-------
| Lysine     | 0.84| 1.02| 1.02| 1.27| 1.01| 0.92| 0.94| 1.20| 1.00 | 6      | 5.84  |
| Histidine  | 0.90| 0.99| 1.01| 0.19| 1.02| 0.23| 2.00| 6    |      |       | 5.89  |
| Arginine   | 3.98| 2.01| 0.34| 1.96| 1.00| 0.90| 0.97| 4.7  | 0.99 | 6      | 5.82  |
| Aspartic Acid | 1.81| 0.94| 1.00| 0.85| 0.90| 0.23| 0.86| 4    |      |       | 3.77  |
| Serine     | 0.83| 1.10| 0.83| 2.90| 2.90| 1.13| 13   |      |      |       |       |
| Glutamic Acid | 4.24| 4.35| 1.02| 1.00| 1.00| 6    | 6.15 | 4    |      |       |       |
| Proline    | 1.01| 0.86| 1.00| 0.93| 0.93| 2    | 2.17 | 2    |      |       |       |
| Glycine    | 3.13| 2.13| 1.00| 1.00| 1.00| 6    | 6.15 | 4    |      |       |       |
| Alanine    | 3.28| 1.00| 0.96| 1.00| 1.00| 6    | 6.15 | 4    |      |       |       |
| Cystic Acid| 1.00| 1.00| 0.86| 0.94| 0.94| 4    | 4.24 | 4    |      |       |       |
| Methionine | 1.00| 1.00| 0.86| 0.94| 0.94| 4    | 4.24 | 4    |      |       |       |
| Isoleucine | 0.87| 0.97| 0.94| 0.97| 0.97| 3    | 2.71 | 3    |      |       |       |
| Leucine    | 2.26| 0.95| 0.99| 1.01| 1.00| 2.07| 8    | 7.94 |      |       |       |
| Tyrosine   | 0.87| 0.97| 0.94| 0.97| 0.97| 3    | 2.80 | 3    |      |       |       |
| Phenylalanine | 0.87| 0.97| 0.94| 0.97| 0.97| 3    | 2.80 | 3    |      |       |       |
| Tryptophan | 2.01| 0.94| 0.99| 1.79| 1.79| 3    | 2.71 | 3    |      |       |       |
| No. of Residues | 21 | 13 | 12 | 4 | 5 | 6 | 9 | 3 | 9 | 82 |
| Purification Procedure | DX | DX | DX | DX | DX | DX | DX | DX | DX | DX | DX | DX | DX | DX |
| Yield (%) | 65 | 50 | 48 | 58 | 41 | 24 | 52 | 48 | 58 |      | 5.84  |

* Values represent the mean result of 20- and 72-hour hydrolysates, calculated on the basis of 1 mole of heme per mole of protein.
* Serum and threonine values are extrapolated to zero hydrolysis time.
* Sample hydrolyzed in the presence of 1% thioglycolic acid.
* Sample hydrolyzed in the presence of 3% thioglycolic acid.

** Isolation and Characterization of Tryptic Peptides of Monkey Cytochrome b$_5$—The amino acid composition of monkey apocytochrome b$_5$ (Table II) was identical with that of the human protein, except for the absence of the characteristic replacement of the single residue of methionine for leucine, and replacement of 1 tyrosyl residue by phenylalanine. Comparison of the tryptic digest elution profiles of these two proteins (Fig. 1, C and B) indicated that the noted composition difference is localized to the sequence region comprised by Peptides T-3 and T-4. Table II summarizes the composition and yields of each peptide isolated from the tryptic digest of the monkey apocytochrome and confirms the above proposed differences between the human and monkey proteins. Since Peptide T-3 contains 1 tyrosyl residue rather than a phenylalaninyl residue as in the case of Peptide T-3 from the human protein, it was evident that this tyrosine represents residue 78. Nevertheless, to check this assignment, the following structure studies were performed on Peptide T-3.

Peptide T-3 (Residues 77 through 90): Thr-Tyr-Lys-Pro-Arg—This peptide was subjected to three steps of the Edman degradation. The results were: Step 1 (81%): Lys, 1.03; His, 0.98; Arg, 0.97; Asp, 2.19; Thr, 0.17; Glu, 1.03; Pro, 1.86; Gly, 1.04; Ile, 1.22; Leu, 0.98; Phe, 0.75. Step 2: PTH-phenylalanine. Step 3 (70%): Lys, 0.99; His, 0.95; Arg, 0.95; Asp, 1.92; Gly, 0.95; Ile, 0.95; Leu, 1.12. As with Peptide T-3 from the calf and rabbit proteins, the presence of 2 isoleucine residues in this peptide is not evident unless the sample is hydrolyzed for 72 hours. Dilute acid hydrolysis of the residual peptide (T-E-3) after the third Edman degradation stage released only 2 eq of aspartic acid, suggesting the presence of an Asp-Lys-Pro-Arg or Asp-Asp-Arg-Pro-Lys COOH-terminal sequence. Gel filtration of this hydrolysate on Sephadex G-25, as described under “Experimental Procedure” gave three peaks. The first peak consisted of the hexapeptide (Ile, 0.90; Gly, 0.02; Glu, 1.12; Leu, 1.14; His, 1.05; Pro, 1.08), representing the expected NH$_2$-terminal segment of the parent peptide. The second peak, a tripeptide, contained the lysyl residue as well as proline and arginine, indicating that it represented the carboxyterminal fragment of T-3, while the third peak was only free aspartic acid. One Edman degradation step on the tripeptide indicated that lysine was amino-terminal. The data were as follows: Composition: Lys, 1.00; Pro, 1.03; Arg, 0.89. Step 1 (yield 51%): Lys, 0.0; Pro, 0.90; Arg, 1.00. From the trypsin specificity it was concluded that arginine is carboxyterminal and is preceded by proline, hence the sequence of this peptide is Lys-Pro-Arg.
Glu, Leu, His, Pro, Asp, Asp, Lys, Pro)—Arg—Three steps of the Edman degradation confirmed the sequence shown above. The results are the following: Step 1 (90%): Lys, 0.85; His, 1.12; Arg, 1.10; Asp, 2.07; Thr, 0.54; Glu, 1.04; Pro, 1.82; Gly, 1.26; Ile, 1.02; Leu, 1.08; Tyr, 0.96. Step 2 (70%): Lys, 1.12; His, 0.80; Arg, 0.77; Asp, 1.98; Thr, 0.36; Glu, 0.80; Pro, 1.70; Gly, 0.67; Ile, 0.72; Leu, 1.00; Tyr, 0.44. Step 3 (50%): Asp, 1.76; Thr, 0.39; Glu, 1.14; Pro, 1.86; Gly, 1.38; Ile, 0.86; Leu, 1.13; Lys, His, and Arg, ND. Characteristic of Peptide T-3 from all other apocytochromes (6, 8) containing isoleucyl-isoleucyl sequence, the isoleucine content of monkey Peptide T-3 increases to 2 residues per molecule only after a 72-hour hydrolysis. Hence, duplicate amino acid analyses were performed only on the initial peptide, and the residual peptide from the last Edman degradation step.

Because the chromatographic mobility (Fig. 1) and the composition (Table II) of all the other tryptic peptides of monkey apocytochromes were identical with those of human or rabbit proteins, and since the sums of the amino acid composition of its tryptic peptides were in good agreement with the over-all composition of the parent protein, the complete primary structure of monkey cytochrome bs must be as shown in Fig. 5.

Isolation and Characterization of Tryptic Peptides of Porcine Cytochrome bs—The elution pattern of the tryptic peptides from a Dowex 1 column is shown in Fig. 1A. This procedure provided peptides accounting for 68 of the 82 amino acids in the porcine cytochrome bs. Similar to the previously investigated apoprotein digests from other species, the nonapeptide (T-12) from the pig protein digest is insoluble in the basic Dowex 1 column elution buffer, and the precipitated peptide may be purified by gel filtration on Sephadex G-25 or may be obtained by Dowex 50 chromatography of the tryptic digest (Fig. 2). As with the calf protein (5), the basic pentapeptide (T-5) is eluted from the Dowex 1 column in a very low yield. The most satisfactory procedure for the isolation of Peptide T-5 is shown in Fig. 2. Thus, for a complete resolution of all the tryptic peptides of the porcine cytochrome bs, the tryptic digest has to be chromatographed on Dowex 1 and Dowex 50 columns concomitantly. The compositions of the tryptic peptides and the yields in which they were isolated are presented in Table III. The sum of the amino acid composition of these peptides accounts for all of the 82 residues of the porcine cytochrome bs. The amino acid sequence of these peptides and their alignment in the parent molecule could be inferred from obvious homologies in composition between these peptides and the tryptic peptides of the calf and rabbit cytochromes. Nevertheless, all of the porcine tryptic peptides were characterized by the conventional procedures.

Peptide T-3 (Residues 7 through 9): Ala—Val—Lys—One Edman degradation step and the tryptic specificity established the sequence of this tripeptide as shown above. The ascending fractions of the Peptide T-8 peak (Fig. 1A) also contained 5 to 10% of Peptide T-5. Edman degradation of these pooled fractions...
ent elution was begun by allowing 80 ml of 8.5 M sodium acetate buffer, pH 5.6, to flow into the mixing chamber containing 80 ml of pH 3.1 buffer. At the fractions indicated by the arrows on the elution diagram, the buffer in the reservoir was replaced by the buffer indicated. Fractions of 1.5 ml were collected at a flow rate of 22 ml per hour, 150 to 300-μl aliquots were analyzed by the ninhydrin method. A, 0.4 μmole of pig apoprotein; B, 0.1 μmole of monkey; and C, 0.3 μmole of human apoprotein.

Peptide T-7 (Residues 10 through 18): Tyr-Tyr-Thr-Leu-Glu-

Peptide T-5 (Residues 19 through 28): His-Asn-Asn-Ser-Lys (Table VI)—Edman degradation established the sequence of the first 3 residues. The remaining 2 residues were positioned from the tryptic specificity. Identification of the phenylhydantoins from the second and third Edman degradation steps established that all aspartyl residues were amidated.

Peptide T-12 (Residues 24 through 32): Ser-Thr-Trp-Leu-Ile-Leu-His-Lys (Table VIII)—Phenylisothiocyanate degradation gave the sequence of the first 6 residues. It is noteworthy that, despite the presence of tryptophan at residue 3, this peptide underwent six Edman degradations without difficulty. The carboxyl-terminal sequence was postulated to be His-His-Lys from the specificity of trypsin.

Peptide T-6 (Residues 33 through 38): Val-Tyr-Asp-Leu-Thr-Lys—The application of three steps of the Edman degradation gave results consistent with the sequence proposed from the composition data. The data were as follows: Step 1 (yield 80%): Asp, 1.00; Thr, 0.90; Val, 0.80; Leu, 0.86; Tyr, 1.04; Lys, ND. Step 2 (78%): Asp, 1.10; Thr, 0.84; Leu, 1.05; Tyr, 0.09; Lys, ND. Step 3 (90%): Asp, 0.22; Thr, 0.84; Leu, 1.04; Lys, ND.

Peptide T-2 (Residues 39 through 51): Phe-Leu-Glu-Glu-His-Pro-Gly-Gly-Glu-Glu-Val-Leu-Arg (Table VIII)—The composition of Peptide T-2 is identical to that of Peptide T-2 from rabbit (8) and calf (5) proteins. Moreover, their electrophoretic mobilities and chromatographic behavior, in the Dowex 1 system, were identical; hence their sequence must be identical and as postulated above. To test the validity of this assumption, Peptide T-2 was subjected to Edman degradation. As summarized in Table VIII, the results of 10 steps of Edman degradation on Peptide T-2 confirm the deduction reached.

Peptide T-1 (Residues 52 through 72): Glu-Gln-Ala-Gly-Gly-Asp-Ala-Thr-Glu-Asn-Phe-Glu-Asp-Val-Gly-His-Ser-Thr-Asp-Ala-Arg (Table IX)—Seven stages of Edman degradation were conducted on Peptide T-1. Four degradations proceeded as expected. In the sixth step the anticipated decrease of aspartic acid content did not take place, and after the seventh stage, the composition of the residual peptide was still identical with that of Stage 5 (cf. Table IX). This suggested that the aspartyl residue at Position 6 was amidated and had undergone cyclization, or that isomerization of this residue to the β peptide form had taken place during the Edman degradations. When Peptide T-1 was subjected to pepsin hydrolysis, three peptides were formed: P-1, P-2, and P-3. These were separated by chromatography on Dowex 50 as described in the legend of Fig. 2, except that the column was developed under a linear gradient established between 400 ml of pH 3.1 buffer and 400 ml of pH 5.6 buffer. Peptide P-1 was present in Fractions 29 to 35 (50 to 60 efficient ml). It was a pentapeptide and had a composition identical with that of the NH₂-terminal segment of the parent peptide. A complete aminoacidic hydrolysis of Peptide P-1 did not confirm that aspartic acid was amidated. Peptide P-2

The results of the Edman degradations on which the amino acid sequences of Peptides T-7, T-5, T-12, T-2, T-4, and T-3 were based (Tables V to X) are available on microfilm. Order NAPS Document 01331 from the National Auxiliary Publications Service, CCM Information Corporation, 900 Third Avenue, New York, New York 10022, remitting in advance $2.00 for microfiche or $5.00 for photocopies (made out to CCM-I-NAPS).
was present in Fractions 39 to 41. Its structure was established by Edman degradations as Ala-Thr-Glu-Asp-Phe. Peptide T-3 (Fractions 49 to 53) contained arginine and therefore is the COOH terminus of T-1. This decapeptide was taken through eight cycles of Edman degradation as shown in Table IX. Hence the complete sequence of Peptide T-1 is determined as written.

Peptide T-4 (Residues 73 through 76): Glu-Leu-Ser-Lys—The amino acid analysis of Peptide T-4 fraction was in accord with the proposed sequence except that it contained 0.34 residue of aspartic acid and 0.19 residue of histidine (cf. Table III). The results obtained from two steps of Edman degradation were consistent with the proposed sequence. The data were as follows: Step 1 (yield, 80%): Lys, 1.13; Asp, 0.31; Ser, 1.10; Glu, 0.05; Leu, 0.98. Step 2 (yield, 60%): Lys, 0.86; Asp, 0.19; Ser, 0.85; Leu, 0.17. Peptide T-5 (Residues 77 through 88): Thr-Phe-Ile-Ile-Gly-Glu-Leu-His-Pro-Asp-Arg (Table Xa)—Edman degradations established the sequence of the first nine amino acids. Hydrolysis of Peptide T-3 with 0.05 M HCl at 110°C for 14 hours released 2 eq of aspartic acid and 1 eq of arginine. Since no other amino acids were released, the COOH-terminal sequence must, therefore, have the sequence as written.

The foregoing results lead to the primary structure for porcine cytochrome b₅ shown in Fig. 5.

Isolation and Characterization of Tryptic Peptides of Chicken Cytochrome b₅—Chicken apoprotein was resistant to tryptic hydrolysis under the conditions which are suitable for the digestion of other apocytochromes. The characteristic turbidity which appears a few minutes after the addition of trypsin failed to disappear during the usual incubation period. Fractionation of other apocytochromes. The data were as follows: Step 1 (yield, 80%): Lys, 1.13; Asp, 0.31; Ser, 1.10; Glu, 0.05; Leu, 0.98. Step 2 (yield, 60%): Lys, 0.86; Asp, 0.19; Ser, 0.85; Leu, 0.17.

Peptide T-7A (Residues 10 through 12): Tyr-Tyr-Arg—Two steps of Edman degradation removed all the tyrosine; therefore, the sequence of Peptide T-7A must be homologous to the 3 NH₂-terminal residues of porcine Peptide T-7.

**Fig. 3.** Gel filtration of tryptic hydrolysate of 0.3 μmole of chicken apocytochrome on a Sephadex G-25 column (60 X 0.9 cm) equilibrated with 30% acetic acid. The sum of the amino acid compositions of these peptides and the yields in which they were isolated are presented in Table IV. The amino acid composition of the parent protein.

**Table IV.**

| Peptide | E-1 | E-2 | E-3 | E-4 |
|---------|-----|-----|-----|-----|
| Lys     | 0.97| ND  | 0.99| 0.71|
| Glu     | 3.13| 2.88| 2.11| 1.06|
| Tyr     | 0.97| 1.06| 0.95| 0.82|
| Leu     | 0.98| 0.99| 0.96| 0.91|

| Peptide | E-5 |
|---------|-----|
| Val     | 0.96|
| Ile     | 2.06|

**Composition:**

| Peptide | E-1 | E-2 | E-3 | E-4 | E-5 |
|---------|-----|-----|-----|-----|-----|
| Lys     | 0.97| 0.99| 1.25| 49% |
| Arg     | 1.01| 1.00| ND  | ND  |
| Asp     | 1.95| 2.08| 2.18| 0.60| 0.48|
| Thr     | 0.96| 0.95| 0.80| 0.90| 0.91|
| Ser     | 1.72| 1.75| 1.94| 1.00| 0.99|
| Glu     | 1.12| 1.27| 1.09| 1.13| 1.01|
| Val     | 1.03| 0.90| 1.14| 0.95| 0.90|
| Trp     | 0.00| 0.98| ND  | ND  | ND  |

**Composition:**

| Peptide | E-1 | E-2 | E-3 | E-4 | E-5 |
|---------|-----|-----|-----|-----|-----|
| Lys     | 0.97| 0.99| 1.25| 49% |
| Arg     | 1.01| 1.00| ND  | ND  |
| Asp     | 1.95| 2.08| 2.18| 0.60| 0.48|
| Thr     | 0.96| 0.95| 0.80| 0.90| 0.91|
| Ser     | 1.72| 1.75| 1.94| 1.00| 0.99|
| Glu     | 1.12| 1.27| 1.09| 1.13| 1.01|
| Val     | 1.03| 0.90| 1.14| 0.95| 0.90|
| Trp     | 0.00| 0.98| ND  | ND  | ND  |

**Composition:**

| Peptide | E-1 | E-2 | E-3 | E-4 | E-5 |
|---------|-----|-----|-----|-----|-----|
| Lys     | 0.97| 0.99| 1.25| 49% |
| Arg     | 1.01| 1.00| ND  | ND  |
| Asp     | 1.95| 2.08| 2.18| 0.60| 0.48|
| Thr     | 0.96| 0.95| 0.80| 0.90| 0.91|
| Ser     | 1.72| 1.75| 1.94| 1.00| 0.99|
| Glu     | 1.12| 1.27| 1.09| 1.13| 1.01|
| Val     | 1.03| 0.90| 1.14| 0.95| 0.90|
| Trp     | 0.00| 0.98| ND  | ND  | ND  |
acid composition that was identical to the parent peptide. It was recovered in 50% yield. The incomplete chymotryptic cleavage of Peptide T-12 can be partly attributed to the low solubility of this peptide at neutral pH. The composition of the second peak (Fractions 24 and 25) again was identical with that of Peptide T-12, but the results of Edman degradations indicated that these fractions consisted of two peptides in about equal proportions. The anticipated chymotryptic cleavage at the tryptophanyl residue afforded these two peptides. Three stages of phenylisothiocyanate degradation were applied to this peptide from trypsin specificity. The presence of tryptophan and an tryptophanyl residue afforded these two peptides. Three stages proportion. The anticipated chymotryptic cleavage at the tryptophanyl residue distal to the tryptophanyl Residue established an Ile-Ile-Val order. The amino acid composition that was identical to the parent peptide. It was recovered in 50% yield. The incomplete chymotryptic cleavage of Peptide T-12 can be partly attributed to the low solubility of this peptide at neutral pH. The composition of the second peak (Fractions 24 and 25) again was identical with that of Peptide T-12, but the results of Edman degradations indicated that these fractions consisted of two peptides in about equal proportions. The anticipated chymotryptic cleavage at the tryptophanyl residue afforded these two peptides. Three stages of phenylisothiocyanate degradation were applied to this peptide mixture. The data were as follows: Composition; Trp, 1.95; Gly, 0.92; Ala, 2.80; Val, 0.92; Phe, 0.99; Ile, 1.90; Leu, 1.15; Phe, 1.15. Step 4: PTH-glycine; acid hydrolysis: Gly, yield, 63%.

Peptide T-3 (Residues 73 through 90): Ala-Leu-Ser-Glu-Thr-Asp-Ile-Glu-Leu-His-Pro-Asp-Arg—The composition of this peptide indicated that it is homologous to the segment in the porcine protein represented by Peptides T-4 and T-3. Six steps of Edman degradations uniquely positioned all the interchanged residues as shown above. The results are the following: Step 1 (yield, 100%): Lys, 1.08; His, 1.08; Arg, 0.80; Asp, 1.92; Thr, 0.95; Ser, 0.88; Gly, 2.35; Pro, 1.95; Gly, 0.92; Ala, 0.20; Ile, 1.50; Leu, 1.50; Phe, 0.92. Step 2 (yield, 100%): Lys, 0.85; His, 0.84; Arg, 0.87; Asp, 2.01; Thr, 0.85; Ser, 0.75; Gly, 2.30; Pro, 1.76; Gly, 0.91; Ile, 1.60; Leu, 1.23; Phe, 1.20. Step 3 (yield, 58%): Lys, 0.77; His, 0.99; Arg, 0.86; Asp, 1.03; Thr, 0.93; Ser, 0.14; Gly, 2.05; Pro, 1.07; Gly, 0.99; Ile, 1.99; Leu, 1.15; Phe, 1.15. Step 4: PTH-glycine; acid hydrolysis: yield, 55%. Asp, 1.93; Thr, 0.84; Ser, 0.21; Gly, 0.89; Pro, 1.99; Gly, 1.01; Leu, 1.24; Leu, 1.19; Phe, 0.97; Lys, His, and Arg, ND. Step 5 (yield, 100%): Asp, 2.03; Thr, 0.56; Ser, 0.22; Glu, 1.42; Pro, 1.62; Gly, 0.88; Ile, 1.80; Leu, 1.00; Phe, 0.95; Lys, His, and Arg, ND. Step 6 (yield, 71%): Asp, 1.92; Thr, 0.26; Ser, 0.15; Glu, 1.28; Pro, 1.85; Gly, 0.89; Ile, 1.90; Leu, 1.00; Phe, 0.20; Lys, His, and Arg, ND. Direct analysis of the dilute hydrolysate of Peptide T-3 on the amino acid analyzer yielded 1.95 residues of aspartic acid and a peak emerging just before lysine. Fractionation of this hydrolysate on a Sephadex G-25 column (cf. Fig. 3) afforded two peptide-containing peaks (Fractions 21 and 27) and free aspartic acid (Fraction 31). Fraction 4 Value from 72-hour hydrolysate.
**Human**

NH$_2$ - Ser

Glu - Ala

Glx

**Monkey**

NH$_2$ - Ser

Glu - Ala

Glx

**Pig**

NH$_2$ - Ala

Glu - Gln

Asn

**Calf**

NH$_2$ - Ser

Glu - Ala

Glx - Gln

Asn

**Rabbit**

Glu - Ala

Asp - Lys - Val - Lys - Tyr - Thr - Leu - Gin - Glu - Ile - Lys - His - Asn - His - Ser - Lys - Ser -

**Chicken**

NH$_2$ - Gly - Arg

Asp - Val - Gin

Asn - Gln

**Human**

**Monkey**

**Pig**

**Calf**

**Rabbit**

Thr - Trp - Ile - Leu - His - His - Lys - Val - Tyr - Asp - Leu - Thr - Lys - Phe - Leu - Glu - Glu - Pro - Gly - Gly - Glu - Glu -

**Chicken**

Ile - Val - Arg - Ile - Ile - Asp

**Human**

**Monkey**

**Pig**

**Calf**

**Rabbit**

Val - Leu - Arg - Glu - Gln - Ala - Gly - Asp - Ala - Thr - Glu - Asn - Phe - Glu - Asp - Val - Gly - His - Ser - Thr - Asp - Ala - Arg - Glu -

**Chicken**

Ile - Val - Arg - Ile - Ile - Asp

**Human**

**Monkey**

**Pig**

**Calf**

**Rabbit**

Leu - Ser - Lys - Thr - Phe - Ile - Gly - Leu - His - Asp - Asp - Arg - Ser - Lys - Leu - Ser - Lys - Pro - Met - Glu - Thr - COOH

**Chicken**

Glu

**Human**

**Monkey**

**Pig**

**Calf**

**Rabbit**

Lys - Pro - Arg - COOH

Lys - Pro - Arg - COOH

Arg - COOH

Ser - COOH

Ile - Thr

**DISCUSSION**

The complete amino acid sequences of the human, monkey, pig, and chicken cytochromes bs are deduced from the results of the tryptic peptide characterization. The total number of residues derived from the compositions of these peptides, listed in Tables I to IV, are in good agreement with the compositions of the parent proteins. The similarities between the tryptic peptides sequenced in this study and those from calf (6, 7) and rabbit (8) cytochromes readily permit alignment of the peptides in a unique continuous order, as delineated in Fig. 5. The sequence of two tryptic fragments from the avian cytochrome further strengthens the assumption that the order of tryptic peptides is identical for all the cytochromes bs. The absence of trypsin-sensitive bonds in the avian protein segments comprised of Peptides (T-5)-(T-12) and (T-4)-(T-3) confirms that they are linked in that order. The deduced linear structures are also in accord with the studies on the NH$_2$-terminal residues of the parent proteins (10). In view of the above findings, and since tryptic hydrolysis did not yield any unanticipated fragments from these cytochromes, characterization of the chymotryptic peptides was not necessary in order to confirm the assigned tryptic peptide order.

An important point of the peptide separations methodology merits discussion: of great value as a means of separating tryptic peptide mixtures of cytochromes bs is the Dowex 1 chromatography, using buffers developed by Rudloff and Braunitzer (18) and Schroeder and Robberson (19). Except for Peptide T-12 and to some extent Peptide T-5, this method affords a very reproducible and sensitive resolution of all the cytochrome bs tryptic digests thus far investigated in this laboratory. The hydrophobic nature of Peptide T-12 led to the necessity for additional procedures such as Dowex 50 chromatography or Sephadex gel filtration for a complete separation of tryptic digests into their components. Peptide T-12 from most cytochromes bs is insoluble in the high initial pH buffer used to develop the Dowex 1 columns, and the precipitated peptide can be readily purified by the gel filtration or by Dowex 50 chromatography. Peptide T-12 from the calf cytochrome, however, is only partially insoluble, and the soluble portion under the usual elution conditions interacts strongly with both the Dowex 1 and Dowex 50.
residues. As already noted elsewhere (7), this factor led to a gap in the initially proposed calf cytochrome b5 sequence. Another peptide segment in which fractionation difficulties are encountered is Peptide T-5. Peptide T-5 with the sequence His-Asn-Aaa-Ser-Lys cannot be identified in the Dowex 1 column eluate, whereas the His-Asn-His-Ser-Lys peptide is recovered in satisfactory yields (cf. Fig. 1). Nevertheless, the nonseverate use of Dowex 1 and Dowex 50 columns together with the use of an amino acid analyzer with sensitivity in the 0.005 μmole range proved to be a very satisfactory means for quantitative recovery and characterization of peptides derived by tryptic hydrolysis of limited quantities of cytochrome b5. Indeed, the foregoing work was accomplished with 0.4 μmole of the human, pig, and chicken protein, and 0.1 μmole of the monkey cytochrome b5.

Of the four cytochrome b5 species investigated in this study, tryptic cleavage of three species proceeded in the anticipated fashion. Tryptic hydrolysis of the chicken apocytochrome proceeded in an unexpected manner. Even after more trypsin was added and the incubation time was prolonged, fractionation of this digest afforded a very low yield of all peptides. A complete hydrolysis of the avian apoprotein was achieved by de-naturing the apocytochrome with urea prior to hydrolysis and performing the reaction in 2 M urea. It should be mentioned that the chicken protein has essentially the same number of tryptic susceptible sites as the other proteins. Indeed, the avian protein has 1 less adjacent acidic residue, at arginyl residue 72, which may even be expected to increase the rate of Peptide T-1 accumulation. Peptide T-1, however, was not found in appreciable yield in this hydrolysate. Such behavior is in contrast to that of the rapidly occurring tryptic hydrolysis at arginyl residues 51 and 72 of the acetylated apocytochrome. A satisfactory interpretation of the increased resistance of the avian apocytochrome to proteolytic digestion must await further research. However, the present observation suggests that the avian apoprotein may have a conformation that resembles that of the native cytochromes b5 which are resistant to tryptic hydrolysis. In this connection it is worth mentioning, as noted earlier in these studies (20), that all lysyl residues in the calf cytochrome b5 are readily exposed to a number of acylating and alkylating reagents. It will be of interest to determine the structural explanation for the elusiveness of these lysyl residues to proteolytic attack.

There are several comments that can be made regarding the sequence of cytochrome b5. First, the hydrophobic residues are fairly uniformly dispersed throughout the sequence. Clusters of more than 2 apolar residues are found only in the segments Trp-Leu-Ile-Leu, and Phe-Ile-Ile. Also of interest is the periodic polar-apolar residue distribution in the segment, residues 30 through 89. The polar-apolar residue sequence here is as follows: 3-2; 2-2; 3-1; 4-2; 3-1; 3-1; 2-1; 5-1; 2-1; and 3-1. It may be suggested that such distribution of hydrophobic residues is likely to be associated with an ordered polypeptide configuration. Another segment of interest is residues 17 through 25. It is entirely devoid of apolar residues. Second, a striking feature present in the cytochrome b5 sequence is the pairing of acidic residues. In the human protein, for example, there are four Glu-Glx, two Asx-Asx, and three Asx, Glx sequences. Interestingly, the remaining 3 aspartyl and 3 glutamyl residues are paired with a hydrophobic residue. Further, essentially all of the lysyl residues are found in the 39-residue NH2-terminal segment. When the cytochrome b5 sequence is examined for evidence of repeating segments, the following examples should be mentioned:

| Table XI |
| Amino acid replacements in six species of cytochromes b5 |
| Amino acid replacement | Inferred base change in coding triplet | Position in sequence |
|------------------------|---------------------------------------|---------------------|
| Lys/Arg                | A/G                                   | 9, 32, 88, 90       |
| Lys/Glu, Glx           | A/G                                   | 6, 17, 76           |
| Lys/Glu                | A/C                                   | 28                  |
| Lys/Ile                | A/U                                   | 17                  |
| His/Asn                | A/C                                   | 21                  |
| His/Tyr                | C/U                                   | 31                  |
| Arg/Thr                | G/C                                   | 12                  |
| Asp/Asn                | G/A                                   | 61                  |
| Asp/Glu                | U,C,A,G                               | 41                  |
| Asp/Ser                | *                                     | 5                   |
| Asp/Ala                | A/C                                   | 7                   |
| Glu/Glu                | G/C                                   | 15                  |
| Glu/Ala                | A/C                                   | 73                  |
| Glu/Ile                | *                                     | 16                  |
| Glx/Ile                | *                                     |                     |
| Thr/Ser                | U,C,A,G                               | 97                  |
| Ser/Pro                | U/C                                   | 89                  |
| Ser/Met                | *                                     | 99                  |
| Val/Gly                | U/G                                   | 8                   |
| Val/Ile                | G/A                                   | 16, 33              |
| Val/Leu                | G/A                                   | 29                  |
| Met/Leu                | A/U,C                                 | 74                  |
| Ile/Leu                | A/U,C                                 | 27, 36, 91          |
| Tyr/Ph                 | U/A                                   | 78                  |

* Denotes two-base changes.

Before considering the structural similarities of the six cytochromes b5 the origin of their different chain lengths should be pointed out. Cytochrome b5 sequences with varying chain lengths probably arose during the isolation stages of the cytochrome, because of differences in the susceptibility of their terminal residue bonds toward the liver peptidases. A number of reports have described the liberation of proteolytic enzymes from lysosomes during the fractionation stages of liver homogenates (21, 22). Although a pancreatic lipase (11) was used to release cytochromes from the endoplasmic reticulum, our attempts to detect proteolytic activity in this lipase preparation, with the bovine cytochrome b5 as a substrate, have been negative. Hence, the rabbit protein has the longest amino acid sequence because of an inherent resistance of its terminal bonds toward proteolytic digestion. Indeed, the Lys-Asp bond in the rabbit protein, residues 6 and 7, is resistant to tryptic hydrolysis, and all the bonds in the NH2-terminal 11-residue segment are resistant to chymotryptic hydrolysis. Moreover, the first seven peptide bonds of this segment are also completely resistant to peptic cleavage. Therefore, the isolation of cytochrome b5 sequence with its true length will depend on the success of con-
trolling endogenous liver proteolysis, or on discovering species
having cytochrome b_6 with terminal segments that are resistant
to such hydrolysis.

Comparison of the primary structures of cytochromes b_6 from
five mammalian and one avian species reveals that in the common
region (residues 4 to 90), 24 residues are variable and 60 are
invariant. Interestingly, the amino acid replacements, particular-
ly among the mammalian cytochromes, are confined to the
terminal segments of the protein. On the whole, the extent of
the residue variance in these sequences is in accord with the
zoological classification. Human cytochrome differs from that
of monkey (Alouatta fuscus) by 2 residues, and from that of
chicken by 15 residues. Of the 15 variant residues between the
pig and the chicken proteins, 6 are basic residues, yet there is a
remarkable conservation of the net charge of the protein. In-
deed, the chicken and the pig cytochromes have an identical
electrophoretic mobility (10). An examination of the amino
acid replacements in terms of the nucleotide interchange for the
codon of each amino acid (Table XI) reveals that all variations
involve a single base change. The three loci involving two-base
changes are among the mammalian cytochrome sequences.
Although there appears to be a preponderance of mutation path-
ways involving adenine to guanine fluctuations, in total there are
equal numbers of transitions (purine/purine, or pyrimidine/
pyrimidine) as of transversion (pyrimidine/purine) mutations.

Because of some missing terminal fragments, presumably con-
taining variable residues, a comparison of the rates of evolu-
tionary variations of cytochromes b_6 and other heme proteins is
premature. Nevertheless, the following statement may be made.
The variance of the cytochrome b_6 sequence is by no means
unique, when compared with that of other heme protein sets, nor
is it identical with that of hemoglobins or cytochromes c, but
agrees with the conclusion drawn from such studies, namely,
that proteins undergo sequence variation in the course of evolu-
tion at very different rates (3). Do the terminal segments in
cytochromes b_6 in general, undergo mutations at a statistically
significantly greater rate than that of other regions? Evidence
on this question must wait until more sequences from different
species are obtained.

In respect to the continuous invariant segment (residues 42 to
72), it is tempting to speculate that it is invariant because the
functional conformation of the protein is critically dependent
on that particular amino acid arrangement. Indeed, the two
imidazole groups, spaced 24 residues apart (residues 43 and 67)
certainly meet the requirements to represent the heme-binding
site of this cytochrome. Hence, mutations in this region may be
lethal not only to the heme binding, but also to the normal
intracellular function and turnover of the protein. Such an
argument, however, would also imply that mutations in the
apparently variant NH_2 and COOH terminal segments do not
result in a significant structure alteration. And then it stands
to reason that a single species should also contain a population
of cytochromes having sequences that vary in these regions.
Consistent with such reasoning is the finding that rabbit cyto-
chrome b_6 is heterogeneous at residues 10 and 90 (8). In the
present studies, however, no evidence of polymorphism was ob-
served. It may indeed be possible that polymorphism of a small
degree could have escaped detection, since only a small quantity
of cytochromes were examined in this study. The results of
current studies on the sequence of cytochrome b_6 components of
an individual human liver, perhaps, will be more decisive.

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Amino Acid Sequences of Tryptic Peptides of Cytochromes $b_5$ from Microsomes of Human, Monkey, Porcine, and Chicken Liver

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