Differences in C-terminal amino acid sequences between erythrocyte and liver cytochrome b_5 isolated from pig and human

Evidence for two tissue-specific forms of cytochrome b_5

Sadao Kimura, Kiyoshi Abe* and Yoshiki Sugita

Department of Biochemistry, Institute of Basic Medical Sciences, University of Tsukuba, and **Tsukuba University College of Medical Technology and Nursing, Sakura, Niihari, Ibaraki 305, Japan*

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Two forms of cytochrome b_5, a soluble erythrocyte form and a membrane-bound liver form, were purified from pig and human, and structural differences between them were analyzed. Porcine and human erythrocyte cytochrome b_5 consisted of 97 amino acid residues and contained the same catalytic domain structure (residues 1–96) as that of the corresponding liver cytochrome b_5, but had one amino acid replacement at the C-terminus (residue 97). These results suggest that erythrocyte cytochrome b_5 is not derived from the liver protein by proteolysis but a translational product from another distinct mRNA of cytochrome b_5.

Liver *Erythrocyte* *Cytochrome* b_5 *Amino acid sequence* *Protein homology* *HPLC*

1. INTRODUCTION

Two forms of mammalian cytochrome b_5 have been reported, a membrane-bound form in liver microsomes [1] and a water-soluble form in erythrocytes [2]. Although the two forms are indistinguishable in catalytic and spectral properties, they differ slightly in molecular mass and amino acid composition [2–5]. To clarify the structural differences between these forms, we have isolated erythrocyte and liver cytochrome b_5 from pig and human, and characterized the tryptic fragments of two forms by various chemical approaches. We report here a structural comparison of tryptic peptides derived from two forms of cytochrome b_5 and the differences in the C-terminal amino acid sequences.

2. MATERIALS AND METHODS

Cytochrome b_5 from porcine and human erythrocytes was prepared as in [2]. The apocytochrome b_5 was obtained by reverse-phase HPLC with a Nucleosil 5C8 column (10 × 250 mm) by a linear gradient of acetonitrile concentration from 0 to 50% in 0.1% trifluoroacetic acid. Detergent-solubilized cytochrome b_5 from porcine and human liver microsomes was purified as in [6]. The preparation of the liver apocytochrome b_5 was obtained by reverse-phase HPLC under the same conditions as described above, except that 70% acetonitrile solution was used instead of 50%. Both erythrocyte and liver apocytochrome b_5 were digested with TPCK-trypsin (Washington) for 16 h at 37°C in 1% ammonium bicarbonate (pH 8.0). The tryptic digests were separated by reverse-phase HPLC with a Nucleosil 5C8 column (10 × 250 mm) or an Ultrasphere ODS column (4.6 × 250 mm). The purified tryptic peptides were hydrolyzed at 110°C for 20 or 24 h with 5.7 N HCl containing 0.1% thioglycollic acid. The amino acid sequences of the C-terminal peptides were determined by the

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manual Edman method. PTH amino acid derivatives were identified by HPLC.

3. RESULTS AND DISCUSSION

The total amino acid composition of cytochrome b_5 was determined by using the hydrolysates of apocytochrome b_5 and its tryptic fragments (table 1). Cytochrome b_5 isolated from porcine and human erythrocytes consisted of 97 amino acid residues. This size is intermediate between those of the detergent-solubilized (unmodified) liver cytochrome b_5 (133 residues) and its trypsin-solubilized protein (about 90 residues) from which the hydrophobic membranous segment was removed. From porcine liver, unmodified cytochrome b_5 was obtained consisting of 133 residues. However, from human liver a cytochrome b_5 was purified that was comprised of 99 residues. Native cytochrome b_5 from human liver which contains the membranous segment was not detected in our preparations. It appears that proteolysis of the native cytochrome b_5 occurs during the purification steps. A similar degradation of rat liver cytochrome b_5 was reported in [7]. The N-terminus of porcine and human erythrocyte cytochrome b_5 was blocked similarly to the corresponding liver proteins and we failed to detect any PTH amino acid derivatives from the purified erythrocyte apocytochrome b_5.

Tryptic peptides of erythrocyte and liver cytochrome b_5 from pig and human were analyzed by HPLC. From porcine erythrocyte and liver proteins, two peptide maps each containing 12 peaks corresponding to the tryptic peptides were obtained (fig.1a). Only two peptides, namely PE6 and PL6, were observed to have different retention times (arrows in fig.1a). Their amino acid compositions also differed, contrasting with the remaining 11 peptide pairs which were identical in terms of retention time and amino acid composition.

Table 1

Amino acid compositions of erythrocyte and liver cytochrome b_5

	Erythrocyte				Liver	
	Porcine	Human	Rabbit[a]	Bovine[b]	Porcine	Human
Asx	10	10	10	9	13	10
Thr	6	6	6	7	11	7
Ser	8	5	7	9	13	5
Glx	16	17	14	16	18	17
Pro	3	6	4	3	4	5
Gly	6	6	6	6	6	6
Ala	6	5	5	5	8	5
Val	4	4	4	4	8	4
Met	0	1	1	0	1	1
Ile	6	4	4	5	9	5
Leu	8	8	9	8	11	9
Tyr	3	3	3	4	4	3
Phe	3	3	3	3	4	3
His	6	7	7	5	7	7
Lys	8	8	10	9	8	8
Trp	1	1	1	1	4	1
Arg	3	3	3	3	3	3
Total	97	97	97	97	133	99

[a] [10]
[b] [5] and unpublished

Numbers of residues per mol are deduced from the averages obtained after hydrolysis of apocytochrome b_5 with 3 M mercaptoethanesulfonic acid (Pierce) for 24, 48, 72 h and by the sum of the residues from tryptic peptides

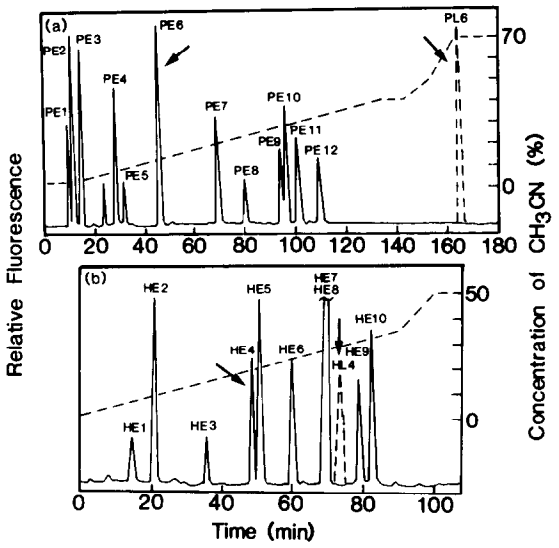

Fig.1. (a) Reverse-phase HPLC of tryptic digests of porcine erythrocyte (——) and liver (– – –) cytochrome b_5. Porcine erythrocyte (7 mg) and liver (4 mg) apocytochrome b_5 were digested with TPCK-trypsin. The tryptic peptides were submitted to reverse-phase HPLC with a Nucleosil 5C8 column and eluted with a gradient of acetonitrile concentration (a) in 0.1% trifluoroacetic acid at a flow rate of 2.0 ml/min. A portion (1%) of the column effluent was diverted to the fluorescamine monitoring system [12]. (b) Reverse-phase HPLC of tryptic digests of human erythrocyte (——) and liver (– – –) cytochrome b_5. Human erythrocyte (1 mg) and liver (2 mg) apocytochrome b_5 were digested with TPCK-trypsin and the digest separated by reverse-phase HPLC with an Ultrasphere ODS column. The column was eluted as described above except that a flow rate of 1 ml/min and 4% for monitoring were used. In both (a) and (b), the tryptic peptides from liver cytochrome b_5 yielded identical retention times with those of the corresponding erythrocyte proteins, except that PL6 (pig) and HL4 (human) were present while PE6 and HE4 were missing in liver samples. The newly observed peptides were added to the erythrocyte maps (– – –).

In the case of human samples, 10 tryptic peptide pairs were obtained (fig.1b). Again, one pair, HE4 and HL4, had different retention times and different amino acid compositions. The remaining 9 pairs were identical. Amino acid compositions of the tryptic peptides of porcine and human erythrocyte cytochrome b_5 were compared with their known liver cytochrome b_5 sequences [8,9]. The peptide orders constructing the whole sequences of, respectively, porcine and human erythrocyte cytochrome b_5 were deduced as PE5-PE3-PE10-PE2-PE11-PE7-PE9-PE8-PE4-PE12-PE1-PE6 (PL6) and HE3-HE7-HE2-HE9-HE5-HE8-HE6-HE1-HE10-HE4 (HL4). In the case of porcine cytochrome b_5, peptide PL6 was composed of 43 residues with a high proportion of hydrophobic amino acids and identified as the membrane anchoring segment (residues 91–133). In contrast, PE6 was found to be a short heptapeptide. The amino acid sequence of PE6 was determined by the manual Edman method and is shown in fig.2. As PE6 lacks a lysine or arginine residue at the C-terminus, it was identified as the C-terminal segment of porcine erythrocyte cytochrome b_5. Serine was found at the C-terminus (residue 97) of PE6, while the corresponding residue 97 in the liver proteins is threonine [8]. The presence of threonine was confirmed by determining the partial sequence of PL6.

In the case of human cytochrome b_5, HE4 was a heptapeptide while HL4 was a nonapeptide. Their sequences were determined and are shown in fig.2 together with those of erythrocyte and liver cytochrome b_5 from rabbit [10] and bovine [5,11] origin. These results indicate that human erythrocyte cytochrome b_5 has one amino acid replacement (Thr → Pro) at the C-terminus (residue 97) compared to that of the liver protein. The results from the rabbit erythrocyte and liver proteins reported in [10] are similar to our findings on the porcine and human proteins in that only one amino acid difference was found at the C-terminus (residue 97). The amino acid sequence of peptide HL4 in human liver cytochrome b_5 was different from the partial sequence (Ile-Thr-Lys,Pro,Ser,Glu,Ser) of human liver cytochrome b_5 reported in [9]. We could not detect any tryptic peptides similar to the reported sequence.

In the case of the bovine protein, erythrocyte cytochrome b_5 has been reported to be identical to the fragment (residues 1–97) of the liver cytochrome b_5 (fig.2) by authors in [5]. They therefore concluded that the bovine erythrocyte cytochrome b_5 is generated from the corresponding liver microsomal protein by proteolytic digestion during maturation of erythrocytes. However, the findings of erythrocyte specific cytochrome b_5 from porcine, human and rabbit sources suggest strongly that bovine erythrocyte cytochrome b_5 is not derived from the liver protein, although it contains

Fig.2. C-terminal sequences of erythrocyte (E) and liver (L) cytochrome b_5. The residues in erythrocyte cytochrome b_5 which differ from the corresponding residues in the liver protein are boxed. a, Here; b, [8]; c, [10]; d, [13]; e, [5] and unpublished; f, [14].

no amino acid replacement. These results suggest that mammalian erythrocyte cytochrome b_5 consists of 97 amino acid residues containing the common catalytic domain structure and lacks the C-terminal membranous segment of the liver protein. Its characteristic structure allows the protein to be soluble in red cells. Furthermore, these results suggest that the two forms of cytochrome b_5 are coded for by two different mRNAs. These conclusions raise the interesting question of how the two forms of cytochrome b_5 are generated in erythrocytes and liver. It is possible that the two distinct mRNAs for the two forms are synthesized from two closely related but different genes. Alternatively, a mechanism generating the two different mRNAs from a single gene may also be possible. Irrespective of the biosynthetic mechanisms, one C-terminal type which characterizes cytochrome b_5 in a specific form, seems to be selected in a tissue-specific manner. The finding of two distinct forms of cytochrome b_5 in erythrocyte and liver provides a good model for understanding the gene expression mechanism in different cell types. Further studies are in progress to clarify the biosynthetic mechanism.

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