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Primary Structure of Cytochrome *c* from the Camel, *Camelus dromedarius**

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ABSTRACT: The amino acid sequence of the cytochrome *c* from heart tissue of the camel, *Camelus dromedarius*, has been established from the structures of the chymotryptic and tryptic peptides. The camel protein consists of a polypeptide chain 104 residues in length with an acylated N-terminal residue. The most remarkable variation in the camel cytochrome *c* is the substitution of valine for the proline commonly present in most mammalian cytochrome *c* in position 44.

A study of the amino acid sequence of cytochrome *c* from camel heart (*Camelus dromedarius*) was undertaken to extend the knowledge of the comparative structures of cytochrome *c* of the "mammalian type" (Nolan and Margoliash, 1968; Smith, 1970) which are all homologous, both structurally and functionally. The isolation procedure, a number of physicochemical properties and the sequence of the heme peptide have been reported elsewhere (Schejter *et al.*, 1972).

Materials and Methods

Cytochrome *c* was prepared as previously described (Schejter *et al.*, 1972). All enzymes were obtained from Worthington. Trypsin was further purified from residual chymotryptic activity by affinity chromatography on Sepharose–ε-aminocaproyl-D-tryptophan methyl ester (Cuatrecasas *et al.*, 1968). Similarly, chymotrypsin was purified by chromatography on Sepharose–ovomucoid inhibitor (Feinstein, 1970). The chemicals used were of analytical grade when available.

Chymotryptic and Tryptic Hydrolysis of Cytochrome c. Cytochrome *c* (96 mg) in 0.2 M NH_4HCO_3 (pH 8.0) at a concentration of 6 mg/ml was digested with chymotrypsin (0.6

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TABLE I: Amino Acid Composition of Chymotryptic Peptides of Camel Cytochrome *c*.

Amino Acid	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI
Aspartic acid	0.1				1.1		2.9					0.2		1.0		1.0	1.1	1.0	1.0	1.1	
Threonine	0.9	0.9					0.8					0.1	0.9			0.8	0.1	0.8			0.9
Serine											0.9										
Glutamic acid	2.1	2.1	1.3	1.0	1.2				1.0	0.2	0.2	0.2		1.0			1.1	1.1	1.1	0.9	
Proline												0.1	0.9			1.1			0.9		
Glycine	1.2	1.2	2.1	1.0	2.0	1.1		1.1			0.1	0.1	1.1	2.1	1.0	1.0	0.8				1.0
Alanine			1.1	0.9		1	1.1			1.0		0.1			1.0		0.9	1.0		1.1	
Valine	0.1		1.1	1.1	0.9	1				0.1				1.0							
Methionine	0.7												0.9								
Isoleucine										1.0		1.0	1.0	1.0	0.9						0.9
Leucine	1.0	1.0	0.5					1.0								1.0	0.9		1.0	0.8	
Tyrosine									0.9	1.0	1.1								1.0		
Phenylalanine			1.0	1.0		1.1		1.0				1.1		0.8							
Lysine					1.8		1.0					0.1	1.0	3.2	1.1	1.1	2.0	2.0	2.1	2.6	1.1
Histidine																0.8					
Arginine																	0.8	0.1		0.8	
Tryptophan																					0.7
Yield (%)	36	16	20	13	25	12	54	66	76	80	70	71	73	50	45	36	40	51	71	10	25
Position	60–65	60–64	41–46	42–46	1–7	43–46	49–54	34–36	66–67	95–97	47–48	81–82	75–80	1–10	83–86	27–33	87–94	99–104	68–74	86–94	55–59

mg/ml) at room temperature for 10 hr. The digest was then lyophilized. Similarly, camel cytochrome *c* (72 mg) was digested with trypsin at 37° for 8 hr. The initial enzyme concentration (0.2 mg/ml) was raised to 0.3 mg after 4 hr, and the reaction mixture was lyophilized after 8 hr.

Isolation and Characterization of Peptides. The peptides were purified on columns (0.6 × 120 cm) of Dowex AG50-X2 resin at 40° with a gradient elution with pyridine–acetate buffers. The Technicon system (peptide autoanalyzer connected to the TSM Model) was used, to sample the effluent and to perform alkaline hydrolyses and ninhydrin analyses. Details of the chromatographic procedures are given in Figures 1 and 2. Peptides isolated from chymotryptic and tryptic hydrolysates are given the prefix C and T, respectively. The homogeneity of the peptides was tested by thin-layer chromatography (tlc) on silica gel G plates with pyridine–acetic acid–1-butanol–H_2O (40:12:60:48, v/v), and by high-voltage electrophoresis in pyridine–acetate (pH 6.4) at 50 V/cm. Mixtures were observed in some cases, but the small amounts of contaminating peptides were readily identified because of the relative simplicity of the mixtures. However, when necessary, as in the case of fractions T-VI and T-XIII, the peptides were further purified by paper chromatography with the solvent used for the tlc.

Sequential Analysis. Analyses were performed primarily by the Edman degradation procedure using trifluoroacetic acid in the cyclization. Quantitation was obtained by the subtractive method (Hirs *et al.*, 1960). C-Terminal analyses were performed with carboxypeptidases A and B at 37° pH 8, 0.05 M Tris-HCl buffer.

Amino acid analyses were carried out on samples hydrolyzed in 1 ml of constant-boiling HCl in evacuated, sealed tubes at 110° for 22 hr. Single-column analyses were performed with the Technicon-TSM Model automatic amino acid analyzer. Tryptophan was determined in the peptides with *p*-dimethylaminobenzaldehyde (Spies and Chambers, 1948).

Results

The amino acid compositions of the peptides obtained from the chromatographic separations (Figure 1) of the chymotryptic digest of camel heart cytochrome *c* are presented in Table I. Similarly, the amino acid compositions of the tryptic peptides (Figure 2) are presented in Table II. To simplify the discussion of the amino acid sequence of the peptides (Tables I and II), the amino acid sequence of camel heart cytochrome *c* is given in Figure 3. Furthermore, characterization of the peptides is summarized in Tables III and IV. We shall limit the discussion only to peptides which have unusual behavior, such as amino acid replacement or unusual cleavage.

Residue 1–10: Acetyl-Gly-Asp-Val-Glu-Lys-Gly-Lys-Lys-Ile-Phe (Peptides CV; CXIV (Table I) and TI and TXI (Table II)). No amino-terminal residue could be identified in the intact protein or in peptides CV and TI by the Edman degradation procedure. Hydrazinolysis of TI (Tuppy and Kreil, 1962) yielded acetyl hydrazide as one of the products. This experiment together with the results listed in Tables III and IV establish the amino acid sequence given above.

Residues 37–46: Gly-Arg-Lys-Thr-Gly-Gln-Ala-Val-Gly-Phe (Peptides CIII and CIV (Table III) and Peptides TIII (Table IV)). Amino acid composition of TXIII have indicated that this fraction is a mixture of peptides. Paper chromatography yielded four ninhydrin-positive spots of which one gave positive Sakaguchi reaction. Its structure was established by Edman degradation: Gly-Arg. Because of the specificity of tryp-

TABLE II: Amino Acid Composition of Tryptic Peptides of Camel Cytochrome *c*.

Amino Acid	I	II	III	IV	V	VII	VIII	IX	X	XI	XII	XIV
Aspartic acid	1.0	0.9	2.1	1.2	1.0	0.2	1.2	1.0				
Threonine			1.8	0.9	1.7	0.2	0.9		0.8		0.7	
Serine			0.8									
Glutamic acid	1.1		1.1	1.2	4.2	0.2				1.2	2.1	
Proline							1.1		1.0			
Glycine	1.0		1.9		1.9	2	2.8	0.4	1.0			0.9
Alanine		1.9	1.9	1.0	0.3						0.9	0.8
Half-cystine											1.8	
Valine	1.1		0.9							1.0	1.1	
Methionine					0.8							0.8
Isoleucine		1.1			0.7				1.1	1.0		1.9
Leucine		1.6			1.6		1.7					
Tyrosine		1.1	0.9		1.0				1.9			
Phenylalanine			1.0				0.9			0.9		1.0
Lysine	1.1		1.1	0.8	2.8	1.1		1.4	1.2	1.8	0.9	2.1
Histidine							0.9				1.1	
Arginine												
Tryptophan					0.8							
Yield (%)	64	40	35	10	20	25	15	52	60	20	55	15
Position	1–5	92–98	40–53	100–104	56–73	23–25	28–37	54–55, 6–7	74–79	9–13	14–22	80–86

sin, free lysine should be released from the parent peptide. Indeed free lysine was found in TXI and therefore may be placed as position 39. The Edman degradation and carboxypeptidase digestions of peptides CIII, CIV, and TIII clearly establish their relative positions in the overall peptide, and importantly the position of valyl residue 44 instead of proline which is the common amino acid residue at that position in most of the mammalian cytochrome *c*. It should be noted that CIII and CIV represent unusual chymotryptic points of hydrolysis at lysyl-39, threonyl-40, and glycyl-41. The leucine found in CIII is present as the free amino acid as determined chromatographically and results probably from the cleavage at position 98. It is important to notice that a chymotryptic peptide covering residue 37–46 was not obtained. However, the fact that unexpected chymotryptic hydrolysis points appeared is not so unusual (*vide infra*). Therefore the sequence of the 37–46 peptide was established as written on the basis of the following evidence: (1) the presence of the 37–40 sequence, in most of the mammalian cytochromes *c;* (2) the appearance of free lysine in the tryptic hydrolysate, although this should be qualified because free lysine might also arise from other positions in the sequence; (3) the agreement between the established overall sequence and the amino acid composition of the protein (Schejter *et al.*, 1972).

Residues 83–94: Ala-Gly-Ile-Lys-Lys-Lys-Gly-Glu-Arg-Ala-Asp-Leu. Edman degradation (four steps) of CXII and CXVII clearly establish the relative position in the peptide. Again

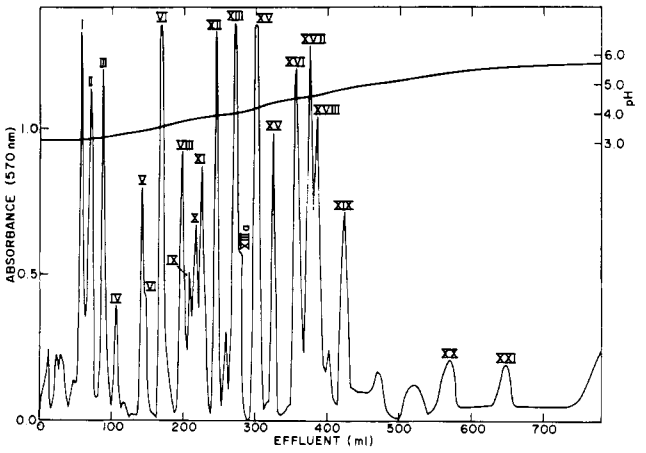

FIGURE 1: Elution profile of the chymotryptic peptides of camel heart cytochrome *c* on a 0.6 × 120 cm column of Dowex 50-X2 at 40°. The column was developed at 30 ml/hr with a gradient of pyridine–acetic acid. Fractions of 3 ml were collected and monitored by ninhydrin analysis at 570 nm after alkaline hydrolysis. The *Roman numerals* being the numbers of the fractions from which pure peptides were obtained. The *straight lines* represent the pH of the eluate.

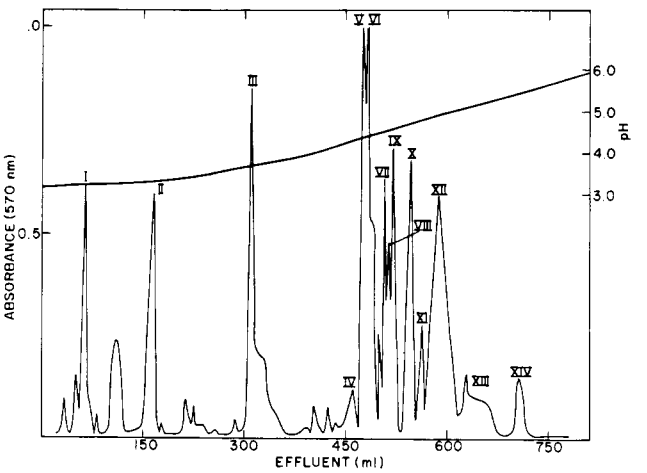

FIGURE 2: Elution profile of the tryptic peptides of camel heart cytochrome *c* on a 0.6 × 102 cm column of Dowex 50-X2 at 40°. Details as in Figure 1.

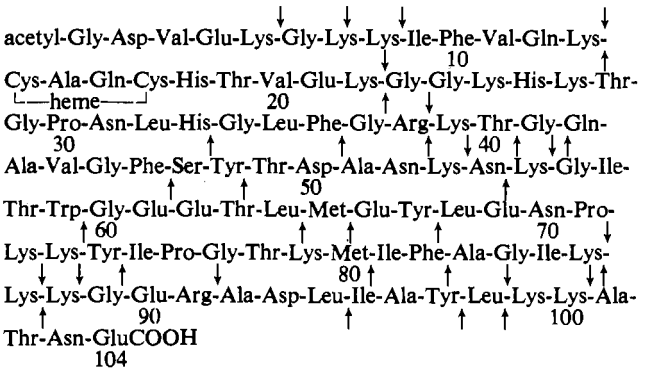

FIGURE 3: Amino acid sequence of camel heart cytochrome *c*. The arrows above the line indicate points of tryptic hydrolysis. The arrows below the line are the points of chymotryptic hydrolysis. Some of the unusual hydrolysis points are indicated (see the text).

tryptic-like activity in the chymotrypsin could result in the formation of the above two peptides. The tripeptide (Gly, 1.01; Glu, 0.92; Arg, 0.93) was obtained from TVI after purification by paper chromatography in 25% yield. The sequence was established by two Edman degradation steps: step 1: Gly, 0.1; Glu, 0.96; Arg, 0.87; step 2: Gly, <0.05; Glu, 0.10; Arg, 0.93.

It should be noted that peptide CXX which was isolated with low yield is similar to CXVII with the addition of another lysyl residue, *i.e.*, this peptide corresponds to positions 86–94.

Discussion

The information obtained with regard to the amino acid sequences of the chymotryptic and tryptic peptides isolated from camel heart cytochrome *c* is sufficient to establish the amino acid sequence of the protein shown in Figure 3. There is no point in elaborating in detail the overlapping peptides since this has previously been discussed for other homologous cytochrome *c* (Margoliash *et al.*, 1961; Matsubara and Smith, 1963; Chan and Margoliash, 1966) from similar, or in many instances, identical peptides. For the most part, hydrolysis of peptide bonds with chymotrypsin and trypsin occurred following those residues which are known to be susceptible to the action of these enzymes. Some uncommon points of chymotryptic hydrolysis were observed at the peptide bonds between residues 22 and 23, and 86 and 89. Similar phenomena have been repeatedly observed with various cytochromes *c* and have been attributed to contamination of the chymotrypsin preparations with trypsin. This assumption would be supported by the fact that other tryptic-like cleavages were observed in this study at residues 38–40. If this was the case then one would expect other tryptic-like cleavages. However no other such points of cleavage were detected suggesting either that these sequences are susceptible to trypsin or that these hydrolyses are in fact due to chymotrypsin. The latter seems to be more likely since the chymotrypsin preparation was freed of trypsin by affinity chromatography (Cuatrecases *et al.*, 1968). Furthermore, recent experiments have indicated that at high salt concentration chymotrypsin has a trypsin-like activity (Y. Shalitin, personal communication). Peptides arising from splits at threonyl residue 40 have been observed previously though in low yields (Needleman and Margoliash, 1966). The unusual tryptic split at the Tyr–Leu bond (97–98) was observed also in the tryptic digests, *e.g.*, of horse (Tuppy and Kreil, 1962) and human (Matsubara and Smith, 1963) cytochrome *c*.

TABLE III: Characterization of the Chymotryptic Peptides.[a]

		Position in the Sequence
Peptide V	(GlyAspValGlu)Lys-Gly-Lys	1–7
Peptide XIV	(GlyAspValGluLysGly)Lys-Ile-Phe	1–9
Peptide XVI	Lys-Thr-Gly-Pro-Asn-Leu-His	27–33
Peptide VIII	Gly-Leu-Phe	34–36
Peptide III	Gly-Gln-Ala-Val-Gly-Phe; Leu (free)	41–46
Peptide IV	Gln-Ala-Val-Gly-Phe	42–46
Peptide XI	Ser-Tyr	47–48
Peptide VII	Thr-Asp-Ala-Asn-Lys-Asn	49–54
Peptide XXI	Lys-Gly-Ile-Thr-Trp	55–59
Peptide I	Gly-Glu-Glu-Thr-Leu-Met	60–65
Peptide IX	Glu-Tyr	66–67
Peptide XIX	Leu-Glu-Asn-Pro-Lys-Lys-Tyr	68–74
Peptide XIII	Ile-Pro-Gly-Thr-Lys-Met	75–80
Peptide XII	Ile-Phe	81–82
Peptide XV	Ala-Gly-Ile-Lys	83–86
Peptide XVII	Lys-Lys-Gly-Glu-Arg-Ala-Asp-Leu	87–94
Peptide X	Ile-Ala-Tyr	95–97
Peptide XVIII	Lys-Lys-Ala(ThrAsxGlx)	99–104

[a] Edman degradations are indicated by (—→) whereas carboxypeptidase A and B hydrolyses are indicated by (←—). The amides were assigned either by the electrophoretic mobility or by the carboxypeptidase hydrolysis.

The primary structure of the camel cytochrome *c* is that of a typical "mammalian-type" cytochrome *c*, namely, a single polypeptide chain 104 residues long, with a blocked amino-terminal residue, and the heme prosthetic group thioether bonded to two cysteinyl residues in positions 14 and 17. The most notable difference between the camel protein and other cytochromes is the substitution of the prolyl residue by a valyl residue.

The differences in sequence between the cytochrome *c* of the hooved animals and the carnivores with which they have a presumed common origin, are found mainly in residues 43, 44, 46, 47, 48, 60, 88, 89, 92, and 103. Of these the changes in the area of 47 are characteristic for the Perissodactyla, of which sequences are available only for the horse and donkey. The Artiodactyla, the order including the suborder, Tylopoda, family Camelidae, all show similar substitutions, partly shared by the horse and the donkey (Dayhoff and Eck, 1969).

It is interesting to point out a unique substitution among the hooved animals at position 44: valine replacing proline. A similar substitution at position 44 has been observed among

TABLE IV: Characterization of the Tryptic Peptides.[a]

		Position in the Sequence
Peptide I	Gly-Asp-Val-Glu-Lys	1–5
Peptide XI	Ile-Phe-Val-Gln-Lys	9–13
Peptide VII	Gly-Gly-Lys	23–25
Peptide VIII	Thr-Gly-Pro-(AspLeuHisGlyLeu)-Phe-Gly	28–37
Peptide III	Thr-Gly-Glx-(AlaValGlyPheSerTyrThrAsx)-Ala-Asn-Lys	40–53
Peptide IX	Asn-Lys; Gly-Lys	54–55 6–7
Peptide V	Gly-Ile-Thr(TrpGlyGlxGlxThrLeuMetGlxTyrLeuGlxAsxProLys)	56–13
Peptide X	Tyr-Ile-Pro-(GlyThrLys)	74–79
Peptide XIV	Met-Ile-(PheAlaGly)-Ile-Lys	80–86
Peptide II	Ala-Asx-Leu-(IleAla)-Tyr-Leu	92–98
Peptide IV	Lys-Ala-Thr-Asn-Glu	100–104

[a] Edman degradations are indicated by (—→) whereas carboxypeptidases A and B hydrolyses are indicated by (←—). The amides were assigned either by the electrophoretic mobility or by the carboxypeptidase hydrolysis.

mammalians so far only in the rabbit protein (Needleman and Margoliash, 1966) which, however, is phylogenetically more distant from the camel than either horse, donkey, or dog. This substitution might be important in terms of the tertiary structure of the protein. The substitution of prolyl to valyl residue involves two base changes in the codon

$$\begin{matrix} A \\ C \\ CCG \\ U \end{matrix} \longrightarrow \begin{matrix} A \\ C \\ GUG \\ U \end{matrix}$$

Therefore, from the evolutionary point of view, it will be of interest to establish the sequences of the cytochrome *c* of the closest animals to the one-humped Arabian camel (*Camelus dromedarius*), *i.e.*, the two-humped Bacterian camel (*Camelus bacterians*) of Turkestan, on one hand, and the llamas of South America, on the other hand.

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