AMINO ACID COMPOSITION OF SOLUBLE TRYPTIC PEPTIDES FROM AMINOETHYLATED BETA-LIKE CHAIN OF RANA ESCULENTA HOMOGLOBIN MAJOR COMPONENT

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1. Introduction

In the last few years several authors have published a number of data on the structure and on some aspects of structure-functional relationship of the hemoglobins from different species of animals pertaining to the Subclass Anoura, Genus Ranidae. Namely Trader et al. [1] have studied the hemoglobin of *Rana grilio*, Baglioni and Sparks [2], Riggs et al. [3], Hamada et al. [4] the hemoglobin of *Rana catesbeiana*, Cristomanos et al. [5] the hemoglobin of *Rana ridibunda*, Trader and Frieden [6] the hemoglobins of *Rana pipiens, Rana grilio* and *Rana catesbeiana*, Chauver and Acher [7] the hemoglobin of *Rana esculenta.*

In previous papers of this series some data have been reported on the structural characterization and on some functional properties of the hemoglobin of *Rana esculenta* L. (Tentori et al. 8–12). The results reported concern both the heterogeneous unfractionated hemoglobin and its two purified components resolved by chromatographic and electrophoretic techniques. It has been shown in detail that the major component of *Rana esculenta* hemoglobin contains four polypeptide chains identical in pairs: one pair of subunits contains a tyrosyl-arginine carboxyl-terminal sequence analogous to that of human hemoglobin α-chain, in the other pair the carboxyl-terminal sequence is represented by tyrosyl-histidine, analogous to those of human β - and δ - chains. The sub-units of *Rana esculenta* hemoglobin have therefore been provisionally denominated α-like and β-like chains. The determination of amino-terminal residues by dinitrophenylaction technique has yelded 3 or 4 dinitrophenyl-glycine residues per mole of hemoglobin major component: it has therefore been deduced that the amino-terminus of both α-like and β-like chains is represented by glycine. These results partly differ from those recently reported by Chauvet and Acher [7]. Using the separated sub-units of *Rana esculenta* hemoglobin major component, the former authors have confirmed our data on the β-like chain, but have found acetyl-alanyl-leucyl as amino-terminal and lysyl-tyrosyl as carboxyl-terminal sequence of α-like chain.

The present paper reports the results of a reinvestigation on amino- and carboxyl-terminus of both α-like and β-like chains and some further data on the primary structure of β-like chain of the major homogeneous component of *Rana esculenta* L. hemoglobin.

2. Material and methods

The preparation of oxyhemoglobin from the blood of adult male frogs (*Rana esculenta* L) and the preparative separation of the two oxyhemoglobin homogeneous components have been performed as previously described [10]. The purity and homogeneity of all the samples have been tested by means of starch-gel electrophoresis with the technique of Smithies [13] and Huisman [14].

The preparation of globin has been accomplished with the method developed by Rossi Fanelli et al. [15].

The preparative separation of polypeptide chains has been attained through the technique of Clegg et al. [16] by chromatography on 2 X 15 cm CM-cellulose columns with phosphate buffer (pH 6.7 containning 8 M urea and 0.05 M mercaptoethanol), using a

molarity gradient from 0.005 to 0.03 M. The purified sub-units have been submitted to aminoethylation by the method of Raftery and Cole [17].

The tryptic digestion of the aminoethylated sub-units has been performed in a pH stat by the technique of Ingram [18]; the peptide patterns both of the trypsin digested samples and of the zones resulting from the chromatographic preparation of peptides, have been studied with the technique of Ingram [18] as modified by Baglioni [19]. The tryptic peptides have been separated on preparative scale with the automatic technique of Jones [20] by chromatography on 1.9 X 15 cm Dowex 50 X 8 (aminex 15) columns. Some of the peaks isolated from tryptic hydrolysates represented mixtures of peptides; in such case the mixtures were resolved into single peptides by rechromatography on 0.9 X 55 cm or alternatively 0.9 X 150 cm Dowex 50 X 2 columns [21] eluted under the conditions described by Jones.

The amino acid composition of the peptides has been determined with the automatic method of Spackman, Stein and Moore [22] after acid hydrolysis with 6 N HCl in high vacuum at 110°C during 24 hr.

The digestion of the sub-units with carboxypeptidase has been performed as described elsewhere [10]. The determination of the amino-terminal residues has been accomplished as previously described [23] after dinitrophenylation of the sub-units by the method of Levy and Li [24].

The quantitative analysis of acetyl groups in the sub-units has been performed by the gas-chromatographic procedure of Ward et al. [25] with a Hewlett Packard gas-chromatograph Series 5750 B on 0.4 X 250 cm columns packed with 10% dimeric acid-silicone oil 550 (1:1 mix.) on silanized Chromosorb W HMDS 60–80 mesh and operated at 100°C.

3. Results

3.1. *Amino-terminal residues*

The dinitrophenylated α-like and β-like chains have been submitted to two different time-periods of acid hydrolysis, namely 6 and 16 hr. No dinitrophenyl-amino acid was recovered by partition chromatography on silica-gel of both 6 hr and 16 hr hydrolysates of α-like chain. The acetyl group content of α-like chain has therefore been determined quantitatively: the gas-chromatography yielded 0.85 acetyl moles per mole of sub-unit thus showing the presence of an acetylated residue in the amino-terminal position of the chain, on the other hand silica-gel chromatography of dinitrophenyl-β-like chain hydrolysates yielded 0.78 residues of dinitrophenyl-glycine per mole of sub-unit after 6 hr hydrolysis, and 0.48 residues of dinitrophenyl-glycine per mole of sub-unit after 12 hr hydrolysis; these data are consistent with the presence of a glycine residue in the amino-terminal posititon of β-like chain. The above results are in agreement with the data of Chauvet and Acher [7] but differ from our data in a previous paper in that we had assigned an amino-terminal glycine to the α-like chain. The discrepancy could be explained by the fact that in the preceeding experiment we had operated on the whole major component of frog hemoglobin instead of on the separated sub-unit.

3.2 *Carboxyl-terminal sequences*

The amino acid recoveries obtained at the end of the digestion of *Rana esculenta* hemoglobin sub-units with carboxypeptidase *A*, carboxypeptidase *B* and carboxypeptidase *A* plus carbosypeptidase *B* are reported in table 1. In the α-like chain, the digestion with carboxypeptidase *A* did not liberate any amino acid, while carboxypeptidase *B* digestion resulted in the liberation of a residue of arginine per mole of sub-unit; finally simultaneous action of carboxypeptidase *A* plus carboxypeptidase *B* released one residue of tyrosine and one residue of arginine per mole of sub-unit. The digestion with carboxypeptidase *A* of the β-like chain resulted in the liberation of one residue of tyrosine and one residue of histidine per mole of the sub-unit while carboxypeptidase *B* had no effect; the kinetics of the experiment suggest that histidine is the first to be released. The results are consistent with the presence of a tyrosyl-arginine terminal sequence in α-like chain and a tyrosyl-histidine terminal sequence in β-like chain, thus confirming our previous results; the difference observed by Chauvet and Acher in the carboxyl-terminus of β-like chain could be accounted for by the existence of different varieties in the species *Rana esculenta*.

3.3. *β-like chain tryptic soluble peptide*

Fig. 1 shows a typical elution pattern of the tryptic hydrolysate of *Rana esculenta* hemoglobin aminoethy-

Table 1
Results of digestion of *Rana esculenta* hemoglobin α-like and β-like chains with carboxypeptidase *A* and *B*.

Chain	Carboxy-peptidase	Time (min)	Number of amino acid residues liberated sub-unit (mol wt 16 000)			
			His	Tyr	Arg	Others
α-like	A	120	-	-	-	-
	B	90	-	-	0.69	0.84
	A + B	120	-	0.78	0.72	1.80
β-like	A	120	0.91	0.87	-	0.74
	B	90	-	-	-	-

lated β-like chain on 1.8 X 15 cm Dowex 50 X 8 column; the roman numerals have obviously been assigned to the zones according to the order of elution; the zones which appeared to contain more than one peptide when tested by fingerprint technique, have been submitted to rechromatography. The resulting isolated peptides have been indicated with the roman numeral of the respective zone, followed by a small letter which indicates the order of elution from the rechromatography column.

Fig. 2 shows the peptide pattern tracing of the tryptic hydrolysate of *Rana esculenta* hemoglobin β-like chain. The roman numerals indicate the correspondence of the spots with the zones of the chromatography and rechromatography of tryptic hydrolysates.

In table 2 the amino acid composition of all the tryptic soluble peptides of *Rana esculenta* hemoglobin aminoethylated β-like chain is reported. The overall

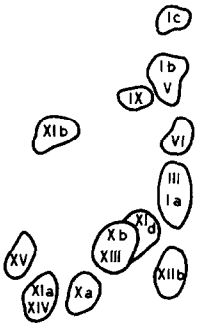

Fig. 2. Tracing of the finger print of aminoethylated β-like chain. The numerals and small letters indicate the correspondence of the spots with the zones of the chromatography and rechromatography of tryptic hydrolysates.

number of residues recovered attains 136; tryptophan is present, but has not been determined in peptide V. Thirteen out of sixteen tryptic peptides are accounted for by ten lysyl and three arginyl residues; two large peptides, namely I c and XIII, contain aminoethyl-cysteine; finally peptide XIb, which does not contain lysine, arginine or aminoethyl-cysteine, has previously been shown to be the carboxyl-terminal of the sub-unit [10]; the sequence of the peptide alanyl-tyrosyl-histidine-COOH, has been deduced from the carboxypeptidase cleavage of the whole chain and confirmed

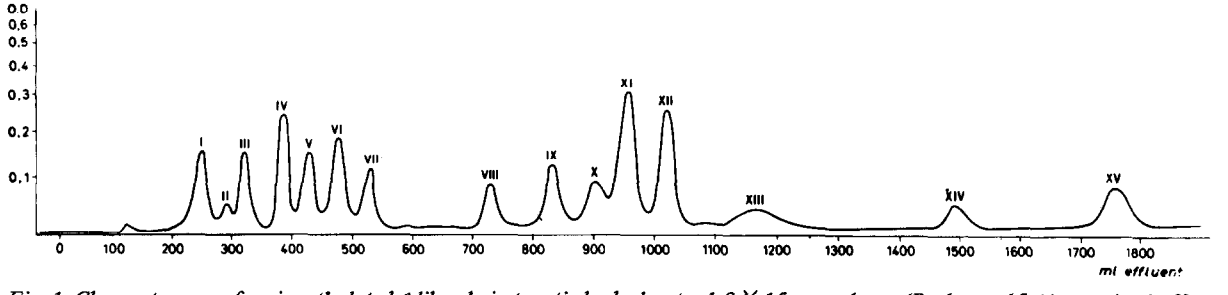

Fig. 1. Chromatogram of aminoethylated β-like chain tryptic hydrolysate. 1.8 X 15 cm column (Beckman 15 A); starting buffer pyridine-acetic acid pH 3.1; limiting buffer pyridine-acetic acid pH 5; 50°C; 120 ml/hr. The gradient has been realized with a Beckman Mod. 131 gradient pump.

by means of carboxypeptidase degradation on the purified peptide.

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