(H) 36

# PRIMARY STRUCTURE OF HEMOGLOBINS :

## (A) Abnormal Human Hemoglobins

## (B) Hemoglobins from Jaguar (Panthera onco) & Leopard (Panthera pardus)

*THESIS SUBMITTED IN FULFILMENT*

*FOR THE DEGREE OF*

**DOCTOR OF PHILOSOPHY**

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UNIVERSITY OF KARACHI

APRIL 1988

*DEDICATED TO MY PARENTS,*

*SISTERS, BROTHERS,*

*MEENO & NADA*

# A B B R E V I A T I O N S

| | |
|---|---|
| CM: | Carboxymethyl |
| DABITC: | 4-N,N-dimethyl amino azobenzene, 4-isothiocyanate |
| DEAE: | Diethylaminoethyl |
| DTE: | Dithioerythreitol |
| FAB: | Fast atom bombardment |
| Hb: | Hemoglobin |
| KCN: | Potassium cyanide |
| $K_3(Fe(CN)_6)$: | Potassium ferricyanide |
| MCHC: | Mean Cell Volume |
| PTH: | Phenylthiohydantoin |
| RP-HPLC: | Reversed phase high performance liquid chromatography |
| SGPT: | Serum glutamic pyruvic transaminase |
| TEMED | N,N,N,N-Tetramethyl-ethylene diamine |
| TFA: | Trifluroacetic acid |
| TIBC: | Total iron binding capacity |
| Tos-Phe-$CH_2$Cl: | (N-Tosyl-L-Phenylalanyl) chloromethane |
| Tp: | Tryptic peptide |
| Tris: | Tris (hydroxymethyl) amino methane. |

# A C K N O W L E D G E M E N T

*I wish to express my deep sense of gratitude to Prof. Zafar H. Zaidi, under whose supervision, continuous help and guidance, I have been able to carry out this work.*

*I am highly indebted to Prof. Gerhard Braunitzer, Department of Protein Chemistry, Max-Planck Institute for Biochemistry, Munich, West Germany for his guidance, help and encouragement and for providing laboratory facilities during my stay in Munich, where part of this work was completed.*

*I am extremely grateful to Prof. Salimuzzaman Siddiqui, F.R.S., H.I., for providing the necessary facilities to work in the field of protein chemistry.*

*I sincerely thank Dr. K. Goeltenboth, Dr. W. Herold and Fatmid Thalassemia Centre for providing the blood samples.*

*I am grateful to Prof. W. Schaefer, Max-Planck Institute for Biochemistry for performing mass spectra of the blocked peptide and Ms. B. Schrank, Mr. K. Mentele, Ms. K. Gautsch and Ms. E. Wottawa for the technical assistance.*

*The financial assistance of the German Academic Exchange Service (DAAD) West Germany (September, 1985 - 1987) is highly acknowledged which enabled me to work in the laboratory of Prof. Braunitzer.*

*I take this opportunity to thank my friends, laboratory fellows, especially Dr. Atiya Abbasi and Dr. Sabira Naqvi for their help and cooperation through out this work. Last but not least to Mr. Mohammad Afzal for his efforts in composing this thesis.*

**AFTAB AHMED**

# A B S T R A C T

The present study on abnormal hemoglobins, jaguar (Panthera onco) and North persian leopard (Panthera pardus) hemoglobins were undertaken to characterize the abnormality in the hemoglobin variants and the primary structure of globin chains respectively.

Different electrophoretic methods i.e. cellulose acetate membrane Polyacrylamide gel disc, isoelectric focussing in thin layer IEF gel and Triton-PAG were used to identify the hemoglobin components and the globin chains.

Hemoglobin components and globin chains were isolated on DEAE-Sephacel and CM-Cellulose in the presence of dissociating agent 8M urea respectively.

Reversed phase HPLC was employed successfully for the isolation of the globin chains and the fingerprinting of tryptic peptides.

The complete primary structures of abnormal peptides and of the globin chains were established by using the liquid and gas-phase sequencer.

Sequence study on abnormal hemoglobins results in the characterization of three variants.

1) Hb-E $\alpha_2{}^{26Glu \rightarrow Lys}\beta_2$ in association with β-thalassemia disorder.

2) A new hemoglobin variant Hb-Karachi $\alpha_2{}^{5Ala \rightarrow Pro}\beta_2$.

3) Hb-Andrew-Minneapolis $\alpha_2{}^{144Lys \rightarrow Asn}\beta_2$ in a German family (Father and his son) from Berlin.

The study on animal hemoglobin resu'ts in the characterization of 6 globin chains i.e. one alpha, and two β-chains in each animal. The sequence alignment with the members of the family Felidae reveals high degree of homology both in the and chains.

C O N T E N T S

## 4. DISCUSSION

# 1. INTRODUCTION

## 1:1. GENERAL INTRODUCTION

Hemoglobin, a pigment associated with a specific physiological function, plays a historic part in chemistry, biology and medicine. The name hemoglobin (synonym from Hämoglobin) was given by Hoppe-Seyler-1864 [1]. It was crystallized by Reichert 1849 [2]. Morphological studies of different hemoglobin crystals were reported by Reichert-1909 [3] showed that the hemoglobin structure is species specific. The molecular mass of hemoglobin was determined correctly by Adair-1924 [4]. Among the eucaryotic proteins, it was the first to be synthesized in a cell free system, which proved that the apparatus for protein synthesis in eucaryots is similar to that discovered earlier in E.Coli by Schweet et al.-1958 [5].

Messenger-RNA for globin was the first eucaryotic messenger to be isolated by Marbaix-1964 [6] and to have its nucleotide base sequence determined by Williamson 1976 [7], Proudfoot and Brownlee-1976 [8].

The physiological significance of hemoglobin as a oxygen carrier was demonstrated by Pfluger-1875 [9].

The primary structure elucidated by Braunitzer et al.-1961 [10] and X-ray structure was reported by Perutz 1960 [11].

## 1.2: RESPIRATORY FUNCTION OF HEMOGLOBIN

Hemoglobin serves as a carrier of oxygen. At high oxygen partial pressure in lungs its heme atom bind to oxygen, which is relibrated at low partial pressure in tissue and brings back the by-product of oxidation, carbondioxide to lungs. This dual function achieved through a reversible change of the s ructure Haurowitz-1938 [12]. The arterial form of hemoglobin has a high affinity for oxygen and low one for hydrogen, chloride ions, carbondioxide and organic phosphate. In the venous form these relative affinities are reversed. Monod et.al.-1965 [13] proposed the theory of allostery to explain the behaviour of enzyme, although hemoglobin is not a enzyme but it follows the same rules. Hemoglobin can be considered as a allosteric molecule in which the substrate is oxygen and the allosteric effectors are proton, chloride and organic phosphate. Perutz-1980 [14] reported that about 50% of alkaline Bohr effects of deoxyhemoglobin depends on hydrogen bond between the carboxy terminal histidine and ß94 (FG1) aspartate of the same chain. 25% of the alkaline Bohr effect results from the salt bridges between the $\alpha$-$NH_2$ group of alpha chain, a 10% from arginines of the neighbouring alpha chain, another 10% Bohr effect is contributed by α122 (H5)His as described by Nishikura-1978 [15]. The Bohr effect also depends on ß143(H21)His and ß144(HC1)Lys. The hemoglobin variants which has substitution at these sites are Hb-Abruzzo [16], Hb-Little Rock [17], Hb-Syracuse [18], Hb-Andrew-Minneapolis [19], and Hb-Mito [20], all have high oxygen affinity and the Bohr effect

is decreased. This observation shows that very minor changes in Bohr effect can bring about a fairly large amount of functional difference in closely related hemoglobins.

## 1.3: HEMOGLOBIN STRUCTURE

Hemoglobin is a respiratory pigment composed of four heme groups and four protein moieties (globin) associated with these, has a molecular mass of 64458. Heme is a complex of an iron atom in a protoporphyrin structure, while globin consist of two pairs of polypeptide chains. The various polypeptide chains namely $\alpha$, $\beta$, $\gamma$ and $\delta$ differ in total number and type of amino acid residues in the chain. The alpha chain has 141 amino acid residues whereas beta, gamma and delta chains each have 146 amino acid residues. The delta and beta chains were found to differ only in 10 residues, the gamma chain however differ from beta chain in as many as 39 residues and contains isoleucine which is absent in alpha, beta and gamma chains.

In normal adult hemoglobin there are two alpha and two beta chains (Hb-A $\alpha_2\beta_2$). During fetal development Hb-F predominates where gamma chains replace beta chains (Hb-F $\alpha_2\gamma_2$). In the minor hemoglobin component Hb-$A_2$, beta chains are replaced by delta chains (Hb-$A_2$ $\alpha_2\delta_2$), is normally found in low concentrations but elevated in certain diseases, such as thalassemia.

### 1.3.1: Perutz model

The low resolution model of horse methemoglobin was reported by Perutz-1960 [11]. The X-ray studies by Bragg et al.-1952a,b, 1954 [21-23] and Perutz et al.-1960 [11] suggested that hemoglobin molecule is spherical of dimensions approx 64x55x50 $\mathring{A}^{\circ}$.

In the oxyhemoglobin, $\alpha$ subunits or $\beta$ subunits have no bonds interconnect to the similar units. The bonds which are formed between the $\alpha$ and $\beta$ produce a $\alpha\beta$-dimers, referred as $\alpha_1\beta_1$-contacts involving B,G and H helices and GH corner are relatively fixed during the transition from oxy to deoxy state of hemoglobin molecule.

The $\alpha_1\beta_2$ interface representing contact between the $\alpha$-subunit of one $\alpha\beta$-dimer and the $\beta$-subunit of the other. Many of these $\alpha_1\beta_2$ contacts involving mainly C, G, helices and FG that present in the oxymolecular conformation are broken with the shift from oxy to deoxy state and a new set of bonds are formed. In the transition to the deoxy conformation the $\beta$ subunit also moves apart from each other is $7\mathring{A}^{\circ}$ forming a cavity to accommodate a molecule of 2,3-diphospho-glycerate.

## 1.4: SYNTHESIS OF HEMOGLOBIN

Heme and globin synthesized de novo in the developing red cell. The synthesis of hemoglobin occurs at the normoblastic stage of development

while the cytoplasm is changing from a basophillic to an orthochromato-phillic colour. A small amount of biosynthesis takes place at the reticulocyte stage; non occur in the adult cell. Most workers agree that synthesis of heme and globin occur independently with slightly prior production of globin. The mRNA is the cytoplasmic templet for globin chain synthesis which serves as a messenger for the genetic information from DNA of the gene. Synthesis of protein occurs on a polysomes group of ribosomes which becomes attached to mRNA and carry the growing peptide chain.. Amino acids are attached to tRNA which is specific for both given amino acid and for group of three mRNA nucleotide i.e. a codon. The amino acyl tRNA bind to appropriate codon of the mRNA by hydrogen bonding. Protein synthesis is achieved by movement of ribosomes along the mRNA strand so that consecutive amino acids are added to the growing peptide chain. The complete chain then associates with other chains to form globin. After conjugation with heme protoporphyrin and formation of quaternary structure an intact hemoglobin molecule emerges.

## 1.5: HEMOGLOBINOPATHIES

The occurrence of genetic disorder has been known to mankind for centuries. As early as 1908, Garrod [24] gave the concept of hereditary metabolic diseases. The discovery of the frequent occurrence of anomalies in structure of human hemoglobin opened another new field of human hereditary abnormalities.

The first hemoglobinopathy to be described was sickle cell anemia Herrick-1910 [25] and thalassemia by Cooley and Lee-1925 [26]. Caminopetros-1938 [27] suggested thalassemia to be a hereditary disease. Elevated fetal hemoglobin (Hb-F) level in the blood of homozygous children at an age when Hb-F has normally disappeared was reported by Vecchio-1946 [28]. Hoerlein and Weber-1948 [29] described a methemoglobinemia, that was characterized by an abnormal absorption spectrum. Sydenstricker-1924 [30] published autopsy report of cases of sickle cell anemia and Wollstein et al.-1928 [31] described the hereditary transmission phenomena of sickle cell anemia. Sherman-1940 [32] discovered that sickle cell hemoglobin crystals are birefringent.

Pauling et al.-1949 [33], found that the isoelectric point of sickle cell hemoglobin (Hb-S) was distinctly different from that of Hb-A.

Barcroft-1928 [34] studied the oxygen binding properties of hemoglobin. The structure of heme group established by Kuester 1913 [35] and Fisher-1927 [36]. Dintzis-1961 [37] discovered that the synthesis of hemoglobin chains on the ribosomes in the cytoplasm was dependent upon the genetic code of the messenger-RNA.

The amino acid sequence of the globin chains from normal hemoglobins alpha, beta, gamma and delta chains were determined [38-40] and thus it was possible to compare the primary structure of different chains.

Kunkel et al.-1957 [41] discovered Hb-$A_2$ and found that the concentration of Hb-$A_2$ increases in the blood of many β-thalassemia hetrozygotes. Pauling-1954 [42], Itano-1957 [43], Ingram and Stretton-1959 [44] reported that the Meditarian form of thalassemia has been due to complete or partial blocking of the synthesis of β-chain. Rigas et al.-1955 [45 and Gouttas et al.-1955 [46] described the occurrence of an unstable hemoglobin in patients with some form of hemolytic anemia. Jones et al.-1959 [47] reported that unstable hemoglobin (Hb-H) is composed of four β-chains. Ager and Lehmann-1958 and Hunt et al.-1959 [48,49] discovered another abnormal hemoglobin in the blood of newborn babies consisting of four gamma chains and named Hb-Barts.

## 1.6: GENETIC ASPECT OF ABNORMAL HEMOGLOBINS

The different forms of hemoglobin found in the red cells of normal individuals are genetically determined and are age dependent. At embryonic stage two hemoglobins namely Hb-Gower-I ($\delta_2\varepsilon_2$) and Hb-Gower-II ($\alpha_2\varepsilon_2$) are present [50], another embryonic hemoglobin Hb-Portland-I ($\zeta_2\gamma_2$) may also be synthesized during this period [51-52]. Hb-F has been identified in the early embryos, its synthesis increases rapidly and by 8 weeks of gestation, Hb-F accounts for at least 90% of the hemoglobin in the erythrocytes. After birth Hb-F synthesis is reduced and correspondingly Hb-A increase, so that by six months Hb-F is usually less than 2% of the total hemoglobin.

At present six different human globin chains are known namely α, β, γ, δ, ε and ζ chains. The synthesis of each chain is under the control of specific structural gene. The genes controlling the synthesis of β, γ and δ chains are aligned very closely on the chromosome, while the gene for α-chain may be on the same chromosome located some distance from the others or even on a different chromosome.

More than one α chain structural loci exist in some individuals. Hollan et al.-1972 [53] described existence of three α chain loci in Hungarian family where some members have two α chain variants Hb-Budha, Hb-G-Pest and normal Hb-A.

Presence of at least two genes for the synthesis of γ chain of Hb-F reported by Schroeder et al.-1968 [54]. The two γ chains differ in position 136 in which an alanyl or a glycyl residue is present, thus resulting in the existence of two fetal hemoglobins i.e. $\alpha_2\gamma_2^A$, and $\alpha_2\gamma_2^G$.

In the β, δ and γ chains the "carboxy terminal" of one gene is next to the "amino terminal" of another. This closeness results in the unequal nonhomologous crossing over between loci resulting in the synthesis of a chain possessing the amino terminal of delta chain and the carboxy terminal of beta chain as in the case of Lepore hemoglobin i.e. delta-beta hybrid (δβ). The reverse case is the anti-lepore the

beta-delta ($\beta\delta$) hybrid. Presence of a hybrid between gamma-beta ($\gamma\beta$) is also known e.g Hb-Kenya [55].

The decreased rate of synthesis of normal polypeptide chains are the hereditary persistance of fetal hemoglobin (HPFH) and the thalassemia. In the HPFH condition there is a failure to switch off the synthesis of gamma chain in adults. Thalassemia are the most common abnormality in the synthesis of specific hemoglobin chain. Since for the production of each globin chain there are individual genes, it is assumed that in the thalassemia specific gene locus is affected. The existence of an alpha, beta, gamma, delta, and delta-beta ($\delta\beta$) thalassemia has been demonstrated. The individual may be heterozygous or homozygous for the thalassemia gene. These conditions are known as thalassemia minor and thalassemia major respectively.

## 1.7: MAMMALIAN HEMOGLOBINS

The red cells of mammalian differ from all other vertebrates because they lack nuclei. The size, shape and life span of red cell vary widely among different species. They appear to be more efficient oxygen carrying unit. All mammalian hemoglobins have substantial Bohr effect [56]. The residues that contribute to the alkaline Bohr effect in human hemoglobin are highly conserved in other mammals. There is small degree of variability in the Bohr effect among mammals. A comparison of the primary structure of more than 50 mammals shows

that the residues involved in physiological activity are highly conserved [57,58].

### 1.7.1: Felidae hemoglobins

Blood from the members of the family Felidae characteristically contains mixture of two or more hemoglobins that can be distinguished from each other by differences in their ability to interact with organic phosphate effector molecules [59]. The difference in the functional behavior between the two type of hemoglobins is attributed to structural alteration that occurs in the β-chain. The component that lack interaction with organic phosphate contain acetylated β-chain amino terminal residue, whereas those that interact, contain free β-chain amino terminal residue.

Two type of hemoglobins were observed in domestic cat and also found in the members from the genera Felis, Acinonyx and Panthera [60].

Bunn et al.-1974 [61] observed that the red cells from cat, leopard, jaguar, hyena, civet and binturong contains low level of red cell DPG and hemoglobin of low oxygen affinity that interacts very weakly with 2,3-DPG.

### 1.7.2: Primary structure of Felidae hemoglobins

The primary structure of hemoglobins from the order Carnivora has not been studied in detail and in particular the family Felidae.

Kleinschmidt et al.-1987 [62] have reviewed amino acid sequences from 12 different species of the order Carnivora. Brimhall et al.-1977 [63] have studied hemoglobin by tryptic mapping. Taketa et al.-1977 [64] reported the fingerprinting of tryptic peptides from cat hemoglobin A and B.

The complete primary structure of hemoglobins from the family Felidae is reported from domestic cat and Amur leopard by Abbasi et al.-1984 [65-66] jaguar and lion by Ahmed et al.-1987 [67] and Jahan et al.-1987 [68] respectively.

## 1.8: EVOLUTION AND PHYLOGENY

Hemoglobin is a protein best studied for molecular evolution. The amino acid sequence of hemoglobin provides information to understand the evolution of this molecule and the degree of relationship.

The investigations on the phylogeny of hemoglobin by its amino acid sequence of related groups of animals has been carried out by many research groups, Braunitzer-1972 [69], Dayhoff et al.-1969 [70], Zuckerkandl-1965 [71]. Goodman-1973 [72], and Fitch et al.-1967 [73].

Goodman et al.-1975 [74] constructed a geneological tree of globin gene by the maximum parsimony method, assuming that the best geneology has the lowest nucleotide substitution. They concluded

that the rate of hemoglobin evolution has been non-uniform, and have put forwarded the view that the amino acid residues involved in $\alpha_1\beta_2$ contact showed many more substitutions at the time of the emergence of jawed vertebrates, when the tetrameric form of hemoglobin was emerging. After the early evolution in the $\alpha_1\beta_1$-contact region these sequence positions have undergone fewer substitution compared with the residue on the exterior of the protein during the vertebrate evolution.

A comparable pattern of fast mutation rates in early cold blooded vertebrates is observed in the globin phylogeny. The rate variations observed using the parsimony method by Goodman et al.-1975 [74] are supported by Holmquist et al.-1976 [75] and Matsuda-1978 [76].

Accelerated α and β chain evolution occurred in lineages of cold blooded vertebrates, marsupials and early placental mammals. The deaccelerated evolution occurred in the two lineages leading to birds and mammals was specially pronounced in higher primates. The rate of evolution was much higher in the reptilian and amphibians that in the lineages to warm blooded vertebrates.

### 1.8.1: Rate of molecular evolution of hemoglobin

Zuckerkandl and Pauling-1962 [77] calculated that 14.5 million years is required for the replacement of one amino acid in a hemoglobin for

another, and that the rate of evolution be calculated by comparing the amino acid sequence of homologous proteins. Later Zuckerkandl and Pauling-1965 [78] reported that the time period required is 7 million years for the substitution of one amino acid residue.

Kirmura-1968,1969 [79-80] proposed the neutral theory of gene mutation in the molecular evolution of proteins. He calculated a value of $1x10^{-9}$ per year for change of each amino acid position for hemoglobin. Dickerson-1971 [81] give the value of 5.8 million year for 1% change in the hemoglobin. These calculation were done on the assumption that the rates of molecular evolution for each protein are almost uniform.

Goodman et al.-1971, 1974, 1975 [74, 82-83] Barnabas et al.-1972 [84] and Moore et al.-1973a,b [85-86] have constructed a geneological tree using maximum parsimony method and have calculated the rate of molecular evolution of hemoglobin in a different way. The mean value obtained by Goodman et al. was 31 NR% (nucleotide replacements per 100 codon per $1x10^{8}$ years), they also proposed that the rate of evolution of hemoglobin is different with each evolution period. They assumed that the period for rapid evolution corresponds to the period when the structure of the hemoglobin molecule was being formed and the slow period corresponds to the time when the structure had become stabilized.

## 1.9: OBJECTIVE OF PRESENT STUDIES

The present work comprises the study on hemoglobinopathies and amino acid sequence of the hemoglobins from jaguar (Panthera onco) and leopard (Panthera pardus).

The objective of the work on abnormal hemoglobins has been carried out to establish the amino acid sequence of the abnormal peptide, which provides the knowledge about the site of mutation and their impact on structure function relationship of the hemoglobin molecule.

The study on amino acid sequence of hemoglobins from jaguar and leopard, representative of the order Carnivora has been undertaken with the intention to establish the complete primary structure of hemoglobin, to fill the gap in the existing sequence of Carnivora hemoglobins. The analysis of primary sequence provide powerful independent information not only on the phylogenetic branch point but also on a rate of molecular evolution.

# 2. EXPERIMENTAL

## 2.1: COLLECTION OF BLOOD SAMPLES

### 2.1.1: Abnormal hemoglobin samples

Abnormal hemoglobin samples for the study of hemoglobinopathies were provided by Fatimid Thalassemia Center Karachi, from two carriers and by Dr. W. Herold, Urban Klinikum, Berlin; from a German carrier.

### 2.1.2: Animal blood samples

The blood samples from the adult Jaguar (Panthera onco) and North Persian leopard (Panthera pardus sexicolor) were kindly provided by Dr. R. Goeltenboth, Zoologischer Garten, Berlin.

## 2.2: ISOLATION OF HEMOGLOBIN

Hemoglobin from heparinized blood was isolated using the procedure of Drabkin–1964 [87]. The blood samples were centrifuged at 4000rpm for 15min. The supernatant plasma was discarded. Erythrocytes were washed thrice with physiological saline (0.9% NaCl). Washed erythrocytes were lysed with bidistilled water (1:1 V/V) and shaken vigorously for 5min. The mixture was kept for 1h in the cold. The cell debris was separated by centrifugation at 4000rpm for 10min. The supernatant containing hemoglobin was transferred and dialyzed against bidistilled water for 5h.

## 2.3: PREPARATION OF GLOBIN

Globin was prepared according to the method of Anson and Mirsky-1930 [88]. A solution of 2% HCl in acetone was cooled to -20°C. Hemolysate was added dropwise to the cooled acidified acetone solution with constant shaking of flask. The precipitated globin was centrifuged and washed twice with cooled acetone. Globin was dissolved in water and traces of acetone removed in vacuo. The solution was dialyzed against bidistilled water for 5h, and lyophilyzed.

## 2.4: ELECTROPHORESIS

### 2.4.1: Polyacrylamide gel disc electrophoresis

Disc electrophoresis of the hemolysate and purified fractions of hemoglobin components was carried out in 10% polyacrylamide gel according to Davis-1964 [89].

**Composition of solutions and buffers**

Sol.A: 36.3g Tris, 48ml 1N HCl, 0.46ml TEMED to 100ml water pH8.9.

Sol.B: 25g Acrylamide, 0.4g Bisacrylamide in 100ml water.

Sol.C: 1g % Ammoniumperoxo-disulphate in water.

Gel was prepared by mixing the following solutions (2ml sol.A, 6.4ml sol.B and 7.1ml water). The solution was mixed and degassed.

0.5ml of sol.C was added to the solution and poured gently in glass tubes (0.5x7.5 cm). The polymerization of the gel was completed in 1h.

About 0.02-0.04mg hemoglobin was dissolved in transformation solution (0.16% KCN and 0.16% $K_3(Fe(CN)_6)$ and one drop of saturated sucrose solution added to it.

Tris/Glycine pH 8.3 was used as electrode buffer. Electrophoresis was carried out for 2h at a constant current of 3.5mA/tube. The gels were stained with 1% amido black-10B in 7% acetic acid and destained with 7% acetic acid.

### 2.4.2: Isoelectric focusing

Isoelectric focusing of the hemolysate was carried out according to Wrigley-1969 [90].

**Composition of solution**

Sol.A: 30g% Acrylamide, 1g% Bisacrylamide in water.

Sol.B: 40% solution of Ampholyte pH 5.5-8.5.

Sol.C: 1g% Potassium persulfate.

Sol.D: 5µl N,N,N',N'-Tetramethyl ethylene diamine (TEMED).

Gel was prepared by mixing 4ml sol.A, 4ml sol.B, 5µl sol.D and 14ml water. The solution was mixed and degassed. 1ml of sol.C was added and poured quickly in a slab (20x20cm) and left overnight to polymerize.

0.4% Ethanolamine and 0.4% sulfuric acid were used as cathode and anode buffers respectively. A potential of 350V (constant) was applied for 3h. The gel was fixed in 12% trichloro acetic acid (TCA) solution. Staining of gel was done with coomassie blue-R250 0.25% in ethanol-acetic acid-water (3:1:6) followed by destaining with a solution of ethanol-acetic acid-water (3:1:6).

### 2.4.3: Triton polyacrylamide gel electrophoresis

Electrophoresis of native globin and separated chains was carried out in 12% polyacrylamide gel in the presence of dissociating agent i.e. 8M urea and a nonionic detergent, Triton X-100 according to Rovera-1978 [91] and Alter et al.-1980 [92].

**Solution for 12% gel**

Sol.A: 30 g% Acrylamide, 0.2g% Bisacrylamide in water.

Sol.B: 2g% Ammonium peroxo-disulphate in water.

**Mixing ratio for gel**

9.6g Urea

8 ml Solution A

1ml Acetic acid

100µl N,N,N',N',-Tetramethyl ethylene diamine (TEMED)

400µl Triton X-100

Total volume made to 20ml with water.

The solutions were mixed and 0.5ml of solution B was added and poured quickly in glass tubes (0.5x7.5cm). Polymerization was completed in 16-18h.

Pre-electrophoresis was carried out at 200V (constant) for 1h. The globin was dissolved in sample buffer (48.5g urea, 10ml acetic acid, 10ml β-mercaptoethanol with water to 100ml). The globin 0.1-0.5mg was loaded on the gel. Electrophoresis was carried out in 5% acetic acid as electrode buffer. A potential of 140V (constant) was applied for 4h. Gels were stained with 1% amido black-10B prepared in 7% acetic acid solution and destained with 7% acetic acid.

## 2.5: SEPARATION OF HEMOGLOBIN COMPONENTS

The hemoglobin components were separated by ion-exchange chromatography as described by Kleinschmidt et al.-1982 [93]. A column (1.6x15cm) was packed with DEAE Sephacel, (wet particle size 40-150mμ, Pharmacia), and equilibrated with 50mM Tris/HCl at pH8.5 containing 0.01% KCN. Hemoglobin was dissolved in the starting buffer and dialyzed over-night against the same buffer. The sample was applied to the column with constant peristaltic pump, eluted by applying a linear gradient of 0.0-0.1M NaCl. Flow rate was maintained at 20ml/h. Absorbance was monitored at 415nm. Pooled fractions were dialyzed against bidistilled water and lyophilyzed.

## 2.6: SEPARATION OF GLOBIN CHAINS

### 2.6.1: Ion exchange chromatography

The separation of globin chains was carried out on a column of CM-Cellulose CM-52, (microgranular; Whatman) according to the method of Clegg et al.-1966 [94].

A column (2.6x15cm) was packed and equilibrated in starting buffer containing 8M urea, 0.025M sodium acetate and 0.2% β-mercaptoethanol pH 5.7. Globin was dissolved in starting buffer, reduced with dithioerythreitol (DTE) under nitrogen for 3h and adjusted to pH5.7. The sample was applied to the column and eluted with a linear gradient of NaCl (0.02-0.1M). Flow rate was maintained at 25ml/h. Absorbance was recorded at 280nm.

Separated globin chains were pooled and desalted by gel filtration on a column of Sephadex G-25 (2.5x150cm), equilibrated with 0.1M acetic acid. Absorbance was monitored at 280nm.

### 2.6.2: Separation of globin chain by RP-HPLC

The reverse phase HPLC was also used for the separation of globin chains. All HPLC separations were carried out on a Beckman gradient liquid chromatograph model 334, controller 421, pump model 110A, UV detector model BT3030 Biotronik, injector from Altex and integrator model CRI-A Shimadzu. The stainless steel columns (4.6x250mm)

supplied by Knauer (Bad Homburg) were filled in our laboratories by column packing machine Gynkothek (Munich). Two different column materials were used.

(1) A column filled with reversed phase material LiChrosorb-RP2 (E.Merck) was equilibrated with 50mM ammonium acetate containing 12% formic acid. Globin dissolved in distilled water was subjected to the column. The elution was carried out with a gradient of acetonitrile 0-35% in 2min., followed by 35-60% in 60min., at a flow rate of 1ml/min. Absorbance was recorded at 280nm.

(2) Hemolysate was separated on a column of Nucleosil-C4 (Macherey and Nagel) equilibrated with 0.1% aqueous trifluoro acetic acid (TFA). Gradient program was the same as used for LiChrosorb-RP2 column. Absorbance was monitored at 230nm.

## 2.7: OXIDATION OF GLOBIN CHAINS

Purified globin chains were oxidized with performic acid by the method of Hirs et al.-1956 [95]. Globin chain 5-10mg was treated with 200µl of a cold solution containing 4.5ml formic acid and 0.5ml hydrogen peroxide. Reaction was stopped after 15min by addition of bidistilled water. Acid was removed in vacuo and the sample lyophilyzed.

## 2.8: ENZYMATIC DIGESTION

The oxidized globin chains were digested with trypsin (EC 3.4.21.4) Tos-Phe-$CH_2Cl$-treated, (Worthington), according to the method of Hirs [96]. The oxidized chain was dissolved in distilled water and the pH adjusted to 10.5 with 12% ammonia. Digestion was initiated by the addition of trypsin in enzyme/substrate ratio of 5:100 for 1h, followed by digestion at pH 9.5 for another 2h. Reaction was stopped by titrating it to pH4 with 2M acetic acid. The mixture was centrifuged at 4000rpm for 10min. The soluble part was subjected to reversed phase HPLC. In case of jaguar hemoglobin mixture of tryptic peptides was prefractionated by gel filtration.

## 2.9: SEPARATION OF TRYPTIC PEPTIDES

### 2.9.1: Gel chromatography of tryptic peptides

Prefractionation of tryptic peptides was carried out on a column (2.6x150cm) packed with Sephadex G-25 fine (Pharmacia). Tryptic digest was applied on the column and eluted with 0.1M acetic acid at a flow rate of 20ml/h. Absorbance was recorded at 230nm.

### 2.9.2: Separation of tryptic peptides by RP-HPLC

The separation of tryptic peptides was carried out by reversed phase HPLC according to the method of Kratzin et al. 1980 [97]. A column of LiChrosorb-RP2 (4.6x250mm) (E.Merck), equilibrated with 50mM

ammonium acetate buffer pH 6.00 was selected for this purpose. Peptides were eluted with a linear gradient of acetonitrile 0-60% in 60min, at a flow rate of 1ml/min. Absorbance was monitored at 230nm. The contaminated peptides were further purified by rechromatography on a Vydac-C18 column (Machery and Nagel) with 0.1% TFA/acetonitrile system.

### 2.9.3: Fingerprinting of tryptic peptides

Fingerprinting of tryptic peptides was carried out on a filter paper sheet according to the method of Ingram-1958 and Baglioni-1961 [98-99]. Tryptic digest was applied on a Whatman No.3 filter paper (38x38cm). The peptides were separated in one dimension by high voltage electrophoresis in 0.02M pyridine acetate buffer pH5. A potential of 2000V was applied for 2h. Descending chromatography was performed in the other direction using solvent system n-butanol-acetic acid-water-pyridine in the ratio of 15:3:12:10. The separated peptides were visualized by stronsium ninhydrin spray as described by Abbasi et al.-1980 [100].

## 2.10: THIN LAYER ELECTROPHORESIS

Purity of separated peptides was checked by thin layer electrophoresis on a pre-coated cellulose plate without indicator, (10x20cm), thickness 1mm (E.Merck). Isolated peptides were dissolved in 0.1M acetic acid, applied on the plate and air dried Plate was sprayed smoothly with

electrode buffer containing 5% pyridine, 5% acetone in water adjusted to pH5.4 with acetic acid. A potential of 450V (constant) was applied for 90min. The plate was dried for 10min. at 80°C. Peptide spots were visualized after spraying with ninhydrin reagent (0.1% ninhydrin, 60% ethanol, 20% acetic acid and 8% collidion) for 10min at 80°C [101]. Presence of His or Tyr was detected after spraying with the reagent described by Frank et al.-[102].

## 2.11: AUTOMATIC AMINO ACID ANALYSIS [103]

About 2-8nmol of tryptic peptides were hydrolyzed in 5.7N HCl under vacuum for 18-20h, at 110°C. The samples were dried in a vacuum concentrator. Tryptophan containing peptides were hydrolyzed in the presence of 6% thioglycolic acid [104]. The peptides with methionine and cystein were determined after performic acid oxidation. The samples were analysed in an automatic amino acid analyzer Model LC5000, Biotronik GmbH. The hydrolysate was dissolved in 0.08M sodium citrate buffer pH2.2. Elution of amino acid was carried out by applying a gradient of pH 3.5-10.5 and temperature from 48°C to 70°C. Amino acids were detected after reaction with ninhydrin reagent. Absorbance was recorded at 570nm and 440 nm.

## 2.12: FAB-MASS SPECTROSCOPY

Amino acid sequence of the blocked peptides were studied by FAB-mass spectroscopy [105]. The peptide FAB spectra of 2KV was recorded

on a mass spectrometer CH7A and MAT 312, Varian (W.Germany) connected with data system SS200/NS.

## 2.13: AMINO ACID SEQUENCING

### 2.13.1: Automatic amino acid sequencing

N-terminal amino acid sequence of the native chains up to 42 amino acid residues and of tryptic peptides was determined by automatic Edman degradation [106] in a liquid phase sequencer Model 890B, 890C, and 890CII, Beckman Instrument, Palo Alto, (U.S.A.) and also in a non-commercial gas phase sequencer [107].

In liquid phase sequencer two different programmes were employed.

**1. Quadrol program**

0.25M Quadrol (N,N,N'N'-tetrakis(2-hydroxypropyl)ethylene diamine was used as a coupling buffer for the sequencing of the intact chain [108], large peptides and lysine peptides. The peptides with lysine were sequenced after modification with reagent IV 7-(Isothiocyanato) naphthalene-1,3,5-trisulfonic acid, trisodium salt [109].

**2. Propyne program**

3-(dimethyl amino)propyne [110] (1.25M) was used as a coupling buffer for the sequencing of arginine peptides, long hydrophobic peptides and lysine peptides having hydrophobic character. Long hydrophobic peptides were reacted with reagent-I 1-(isothiocyanato)benzene-4-

sulfonic acid, sodium salt [111] prior to sequencing. Conversion into phenylthiohydantoin derivatives of amino acid was performed in an autoconverter sequmat PG (Kontron Technik), with 3M TFA at 60°C.

### 2.13.2: Identification of PTH amino acid

About 1nmol of PTH derivatives of amino acid was analysed on HPLC 1084B-liquid chromatograph (Hewlett Packard) at 60°C. Two different programs were used for the identification of PTH-amino acid.

**Stepwise gradient elution [112]**

A column 4.6x250mm filled with ODS-Hypersil-RP18 (Shandon) was equilibrated with 10mM ammonium acetate pH 4.8. Sample was eluted with a gradient of acetonitrile.

| Gradient: | Time | %B |
|---|---|---|
| | 0 min. | 10 |
| | 1 " | 20 |
| | 5 " | 45 |
| | 14 " | 70 |
| | 19 " | 10 |

Detection was monitored at 265nm and 330nm.

**Isocratic elution [113]**

PTH-amino acid also eluted isocratically on ODS-Hypersil-RP18 column at 60°C. Elution was carried out with a buffer containing 1M sodium acetate pH5.3-acetonitrile - 0.5% dichloroethane-water (9:330:7:670). Detection was carried out at 265nm, serine and threonine were identified as their dehydro derivatives at 330nm.

### 2.13.3: N-terminal sequence analysis by DABITC

Amino acid sequence of native chain was determined with DABITC manual degradation method according to Chang et al.-1978 [114] and von Bahr Lindstrom et al.-1982 [115]. Native chain was dissolved in 80µl 50% aqueous pyridine and treated with 40µl DABITC solution (2mg/ml pyridine). Tube was flushed with nitrogen and heated at 50°C for 45min., this was further treated with 10µl PITC and kept at 50°C for 30min. The excess reagent was removed by extracting thrice with 0.5ml heptane/ethyl acetate (2:1). The aqueous phase was evaporated to dryness. The dried material was dissolved in 50µl anhydrous TFA, flushed with nitrogen and heated for 15min. The acid was then removed under vacuum and the sample dissolved in deionized water. The cleaved thiazolinone derivative is extracted twice with 50µl butylacetate. The water phase was evaporated and used for the next cycle.

The butylacetate extract was evaporated and dissolved in 30µl TFA (50%) and heated at 50°C for 50min. The cyclized material was dried and dissolved in 5µl ethanol for TLC identification.

### 2.13.4: Identification of DABTH-amino acid

The DABTH-amino acid was identified on polyamide plate (3x3cm) from Chang Chin Co. (Taiwan) by two dimensional chromatography. The solvent system 33% acetic acid and toluene-hexane-acetic acid (6:4:3) were used respectively. The plate was dried and exposed to HCl vapors. The DABTH derivatives identified by development of various coloured spots relating their positions with the marker.

#### Preparation of marker

Marker was prepared by mixing the following solutions:

Sol.A: 30µl diethylamine, 300µl (50%) pyridine and 150µl DABITC solution.

Sol.B: 60µl ethanol amine, 300µl (50%) pyridine and 300µl DABITC Solution.

DABITC Sol: 0.8mg DABITC in 450µl pyridine.

Solutions were heated at 50°C for 1h, dried under vacuum and 400µl ethanol added to each tube. Both solutions were then mixed in equal concentration and used as marker.

# 3. RESULTS

## 3.1: HEMOGLOBIN E β-THALASSEMIA

### 3.1.1: Clinical examination

Blood from propositus, a 15 year old boy, was analyzed for hematological and biochemical examination, which revealed the following:

Hemoglobin 6.39g/dl, reticulocytes 11%, PCV 0.24l/l, MCHC 26g/dl, bilirubin total 4.2mg/dl, direct 2.6mg/dl, serum iron 220 g/dl, TIBC 320μg/dl, SGPT 130U/l and alkaline phosphates 386U/l. Hepatitis B surface antigen (Australia antigen) was nonreactive. The morphology of erythrocytes showed severe hypochromia, aniso-schisto and poikilocytosis. Film suggestive of thalassemia.

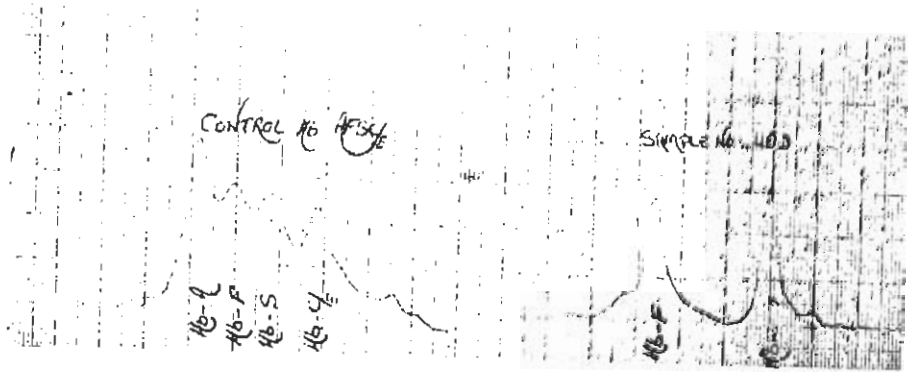
### 3.1.2: Radiological examination

Radiological examination of the skull, both hands, wrist, radius ulna, pelvis, both hip joints and both femora showed the markedly generalized osteoporosis and demineralization. The vault bone dipole showed expansion. Slightly expansion in the schaft of metacarpal and little cystic changes observed in the first and 5th metacarpal of both hands.

### 3.1.3: Electrophoresis

Hemolysate analysed by electrophoresis on cellulose acetate membrane showed an abnormal hemoglobin band at position of Hb-$A_2$, about 55%, and an elevated Hb-F making up for the rest. No hemoglobin band was observed for Hb-A. The densitometric pattern of hemoglobins is presented in Fig.1.1.

Figure 1.1: Densitometric pattern of hemoglobin (Hb-E). Electrophoresis on cellulose acetate membrane, electrode buffer Tris-EDTA-borate pH8.4



Disc electrophoresis of hemolysate on 10% polyacrylamide gel at pH 8.3 (Fig.1.2) showed similar pattern, as obtained by membrane electrophoresis.

### 3.1.4: Chromatography on DEAE-Sephacel

The hemolysate applied on the column of DEAE-Sephacel resulted in the separation of two hemoglobin components. Separation profile obtained is presented in Fig.1.3.

### 3.1.5: Reversed phase HPLC of globin chains

Separation of abnormal chain was achieved by reversed phase HPLC on a column of Nucleosil-C4 (Fig.1.4.). The elution profile showed only an abnormal β-chain followed by α and γ-chains. The normal $\beta^A$ chain was not found. Presence of small amount of δ chain was also observed in pre α-region.

### 3.1.6: Reversed phase HPLC of tryptic peptides

The tryptic digest of abnormal chain was separated by fingerprinting on reversed phase HPLC. The separation profile revealed two abnormal peaks identified as βTp3a and βTp3b and absence of a peak for normal βTp3 (Fig.1.5).

Figure 1.2: Disc electrophoretic pattern of hemoglobin (Hb-E) on polyacrylamide gel.
Tris/Glycine buffer at pH8.3

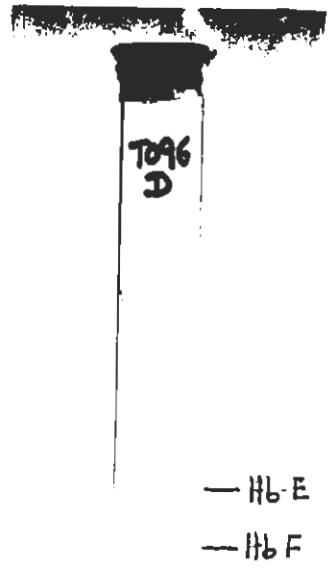


Figure 1.3: Separation profile of hemoglobin (Hb-E) on DEAE-Sephacel (1.6x15cm) column.
Elution buffer: 0.05M Tris/HCl containing 0.01% KCN pH8.5

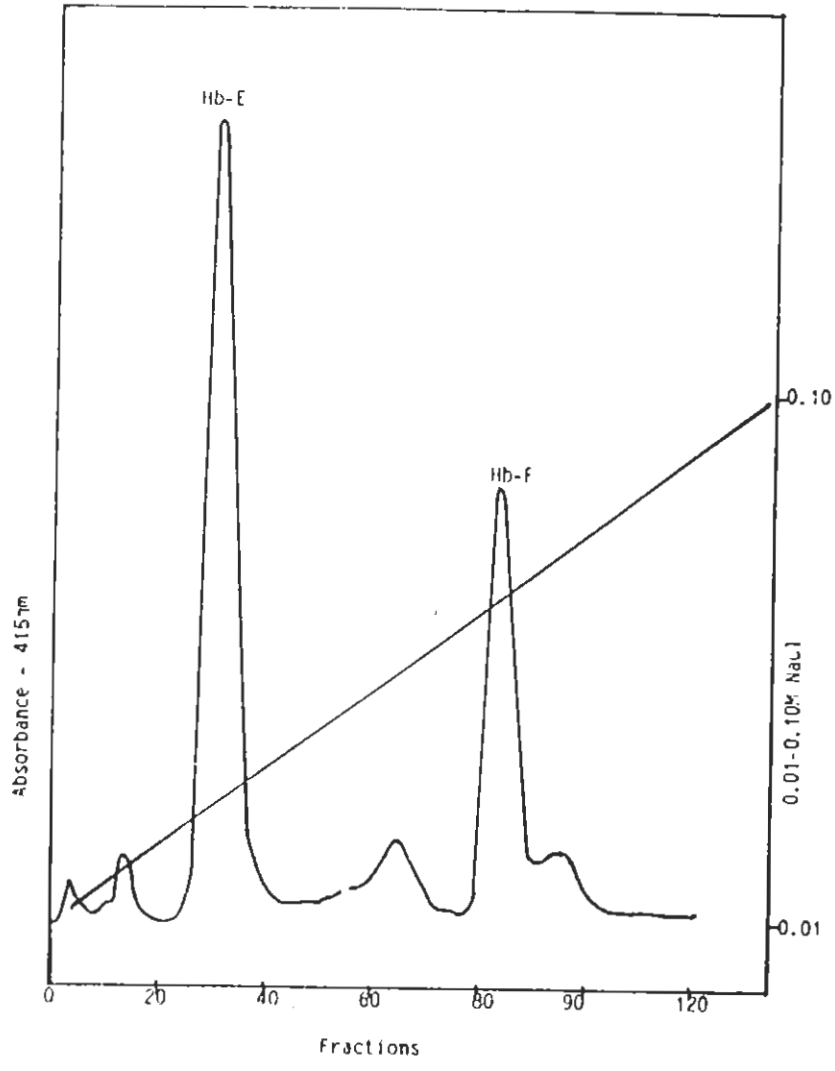


Figure 1.4: Elution pattern of globin chains from Hb-E on RP-HPLC column of Nucleosil-C4 (7µm).
Eluted with 0.1% TFA; gradient from 0-35% acetonitrile in 2min, 35-60% in 60min; flow rate 1ml/min.

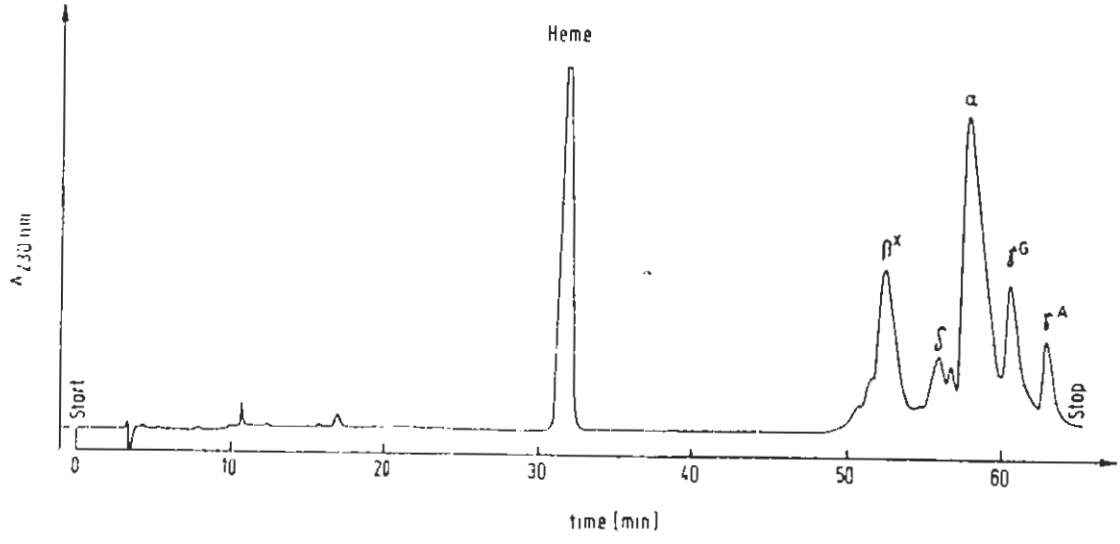
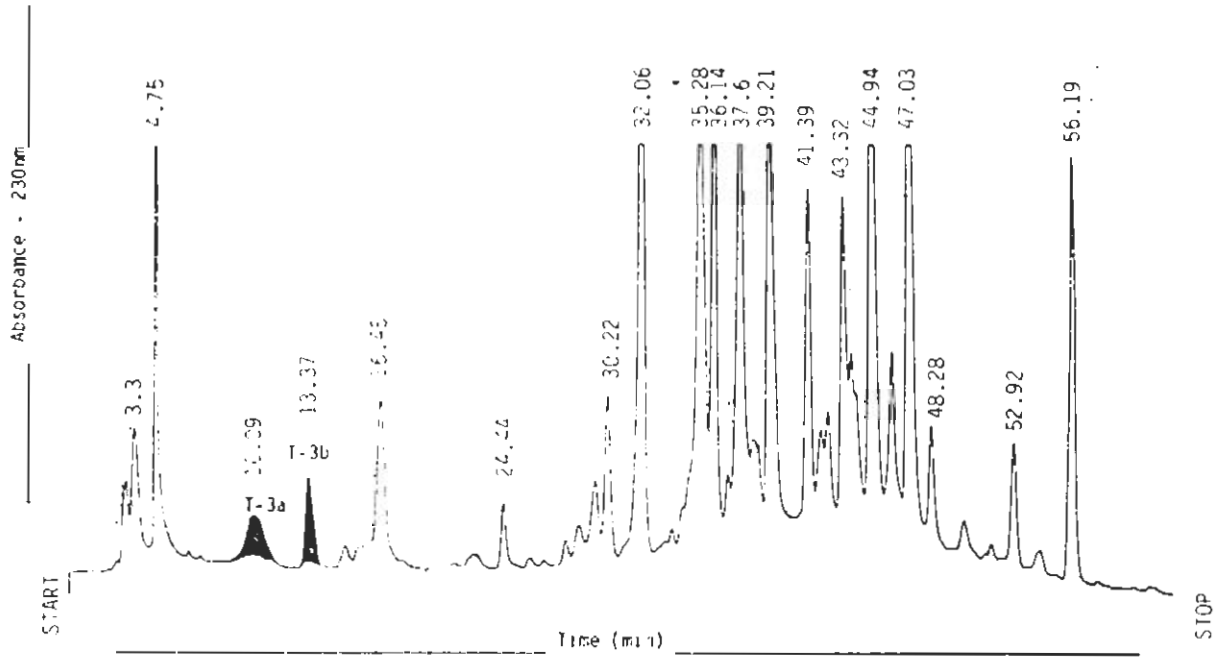


Figure 1.5: Separation of tryptic peptides from the oxidised β-chain (Hb-E) by RP-HPLC. The abnormal peaks are shaded. Column: LiChrosorb-RP2 (7μm); buffer 0.05M ammonium acetate pH6.0; gradient 0-60% acetonitrile in 60min; flow rate 1ml/min.



### 3.1.7: Amino acid analysis

The abnormal peptides obtained by fingerprinting of tryptic peptides were analyzed for their amino acid composition. The amino acid composition is presented in Table 1.1.

### 3.1.8: Amino acid sequence

Amino acid sequence of the abnormal peptide was determined in liquid-phase sequencer, which revealed the substitution of glutamic acid with lysine at position β26 (B8). The results confirm it as a case of Hb-E.

| | 19 | | | 23 | | | 27 | | | 31 |
|---|---|---|---|---|---|---|---|---|---|---|
| $\beta^{A}$Tp3 : | Val | Asn | Val | Asp | Glu | Val | Gly | **Glu** | Glu | Ala | Leu | Gly | Ala |
| $\beta^{E}$Tp3a: | Val | Asn | Val | Asp | Glu | Val | Gly | **Lys** | | | | | |
| $\beta^{E}$Tp3b: | Glu | Ala | Leu | Gly | Ala | | | | | | | | |

Table 1.1: Amino acid composition of abnormal peptide. Numbers in parentheses denote amino acid residue found during sequencing.

| Amino acid | $\beta^{E}$Tp3a | $\beta^{E}$Tp3b | $\beta^{A}$Tp3 |
|---|---|---|---|
| Asp | 1.97 (2) | - | (2) |
| Glu | 1.02 (1) | - | (2) |
| Gly | 2.03 (2) | 0.99 (1) | (3) |
| Ala | - | 1.05 (1) | (1) |
| Val | 2.98 (3) | - | (3) |
| Leu | - | 1.01 (1) | (1) |
| Lys | 0.98 (1) | - | - |
| Arg | - | 0.93 (1) | (1) |
| Sum | 9 | 4 | 13 |

## 3.2: HEMOGLOBIN KARACHI

### 3.2.1: Clinical examination

Hematological and biochemical examinations of propositus revealed the following:

Hemoglobin 15.2g/dl, red cell count $5.1x10^{-12}$/l, reticulocytes 0.5%, PCV 0.47l/l, MCV 92fl, MCHC 32g/dl, total bilirubin 1.3mg/dl serum iron 106 g/dl. Erythrocytes morphology was normal. Incubation of fresh red cells with brilliant cresyl blue disclosed no inclusion bodies within the red cells. The osmotic-fragility test revealed 50% hemolysis at 0.43% NaCl, being within normal range. Heat stability test gave normal results.

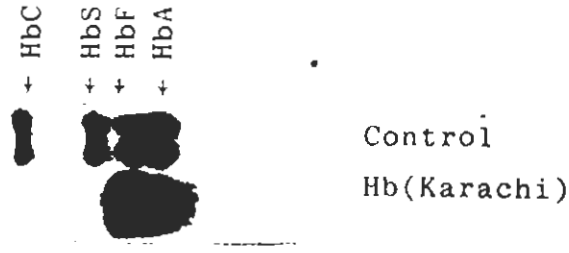
### 3.2.2: Electrophoresis

Hemolysate from propositus, his mother and two sisters was analysed on cellulose acetate membrane in Tris-EDTA-Borate buffer at pH 8.4. An abnormal hemoglobin band migrating more towards cathode than Hb-A was observed in the hemolysate of propositus (Fig.2.1). Neither the mother, nor the two sisters of the propositus showed any abnormal band in electrophoresis of their hemoglobin.

### 3.2.3: Chromatography on CM-Cellulose

The chromatography of crude globin on CM-Cellulose (CM-52) in the presence of 8M urea resulted in the separation of globin chains

Figure 2.1: Electrophoretic pattern of hemoglobin Karachi on Cellulose acetate membrane. Electrode buffer Tris-EDTA-borate pH8.4



(Fig.2.2). The first peak contained unbound material followed by the β and α globin chains.

### 3.2.4: Fingerprinting of tryptic peptides

The fingerprinting of tryptic peptides from the α-chain showed a change in its position of peptide Tp1 (Fig.2.3).

### 3.2.5: Amino acid analysis

Amino acid composition of native chain and of peptide αTp1 showed an elevated value for proline. The composition of peptide αTp1 is presented in Table 2.1.

### 3.2.6: Amino acid sequence

The N-terminal amino acid sequence of abnormal α-chain was established by DABITC manual degradation method upto 10 residues and by automatic method in a liquid-phase sequencer upto 30 residues. It revealed that alanine at α5(A3) is substituted with proline. There was no trace of Ala in this position suggesting that the individual has homozygous variant.

| | 1 | | | | 5 | | | | | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Hb Karachi: | Val- | Leu- | Ser- | Pro- | **Pro**- | Asp- | Lys- | Thr- | Asn- | Val |
| Normal $_{\alpha}^{A}$ : | Val- | Leu- | Ser- | Pro- | **Ala**- | Asp- | Lys- | Thr- | Asn- | Val |

Figure 2.2: Separation of polypeptide chains from Hb-Karachi on CM-Cellulose column.

Elution buffer: 8M urea, 0.2% mercaptoethanol and 0.01M $NaH_2PO_4$; gradient pH 6.5-7.2.

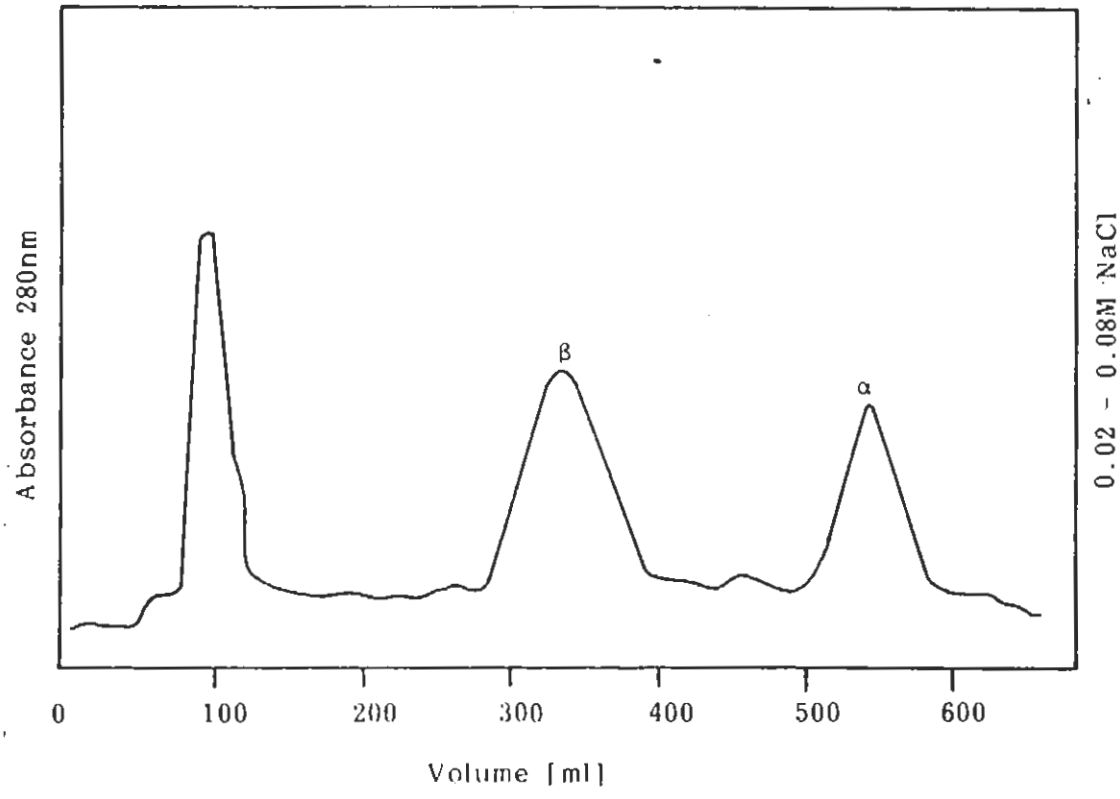


Figure 2.3: Fingerprint of the tryptic peptides of abnormal α-chain (Hb-Karachi) on Whatman filter paper No-3.

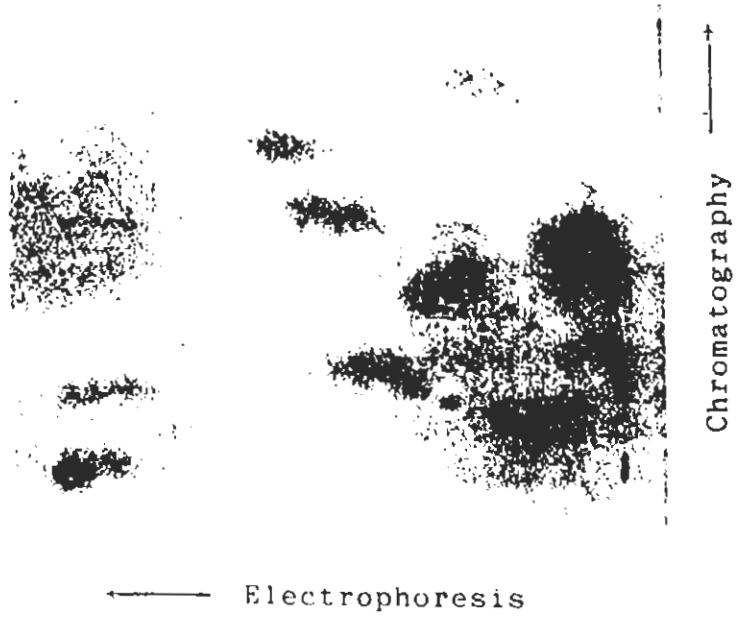


Table 2.1: Amino acid composition of abnormal peptide. Numbers in parentheses denote amino acid residue found during sequencing.

| Amino acid | $\alpha^{X}$Tpl | $\alpha^{A}$Tpl |
|---|---|---|
| Asp | 0.85 (1) | (1) |
| Ser | 0.70 (1) | (1) |
| Pro | 1.76 (2) | (1) |
| Ala | - | (1) |
| Val | 0.83 (1) | (1) |
| Leu | 0.82 (1) | (1) |
| Lys | 0.78 (1) | (1) |
| Sum | (7) | (7) |

## 3.3: HEMOGLOBIN ANDREW-MINNEAPOLIS

### 3.3.1: Clinical examination

Blood samples of propositus and his family members were analysed to determine the hematological parameters, are presented in Table 3.1.

### 3.3.2: Electrophoresis

Isoelectric focusing of hemolysate on thin layer IEF gel in the pH range; 5.5-8.5, showed two abnormal bands in the hemolysate of father and one of his son. These abnormal bands migrated more towards anode than Hb-A. IEF patterr of hemolysate from propositus and his family members is shown in Fig.3.1.

Polyacrylamide gel disc electrophoresis of hemolysate revealed only one abnormal hemoglobin band migrating more towards anode than Hb-A (Fig.3.2a).

Analysis of native globin by polyacrylamide gel electrophoresis in the presence of dissociating agents i.e. 8M urea and Triton X-100 revealed an abnormal β chain shown .n Fig.3.2b.

### 3.3.3: Chromatography on DEAE-Sephacel

The separation of hemoglobin components was achieved by ion-exchange chromatography on DEAE-Sephacel. The separation profile (Fig.3.3)

Figure 3.1: Isoelectric focusing pattern of hemoglobin. ucb= Umblical cord, 1= father (abnormal Hb-Andrew-Minneapolis), 2= son I, 3= wife, 4=son II, 5= daughter.

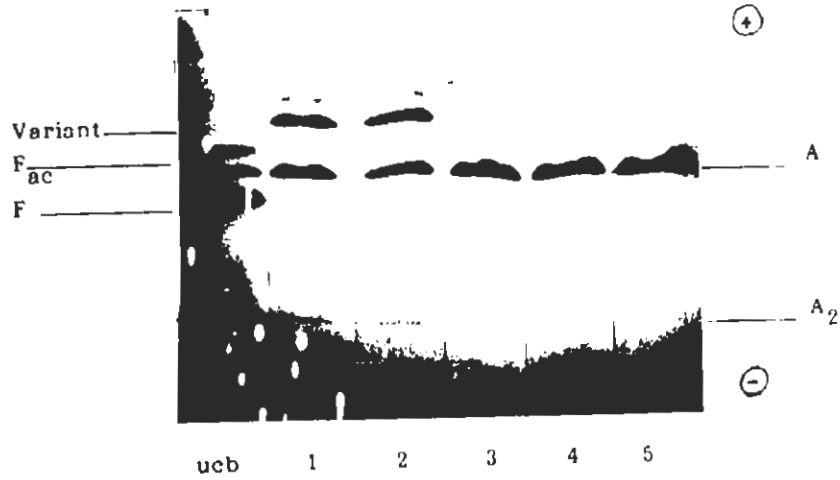


Figure 3.2: Disc electrophoretic pattern of propositus hemolysate (Hb-Andrew-Minneapolis) on polyacrylamide gel.
a) Disc at pH8.3 b) Under dissociating condition 8M urea and Triton X-100.

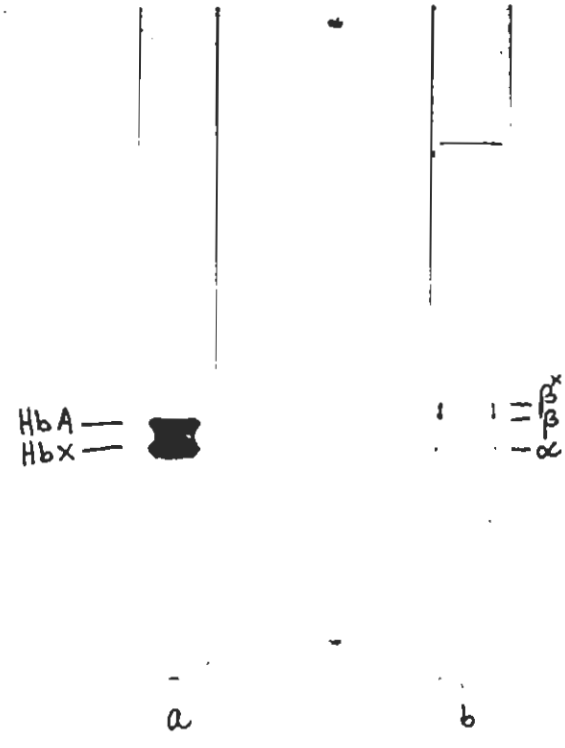


Figure 3.3: Separation profile of propositus hemoglobin (Hb-Andrew-Minneapolis) on DEAE-Sephacel column (1.6x15cm). Eluted with 0.05M Tris/HCl containing 0.1% KCN pH8.5; gradient NaCl 0-0.1M.

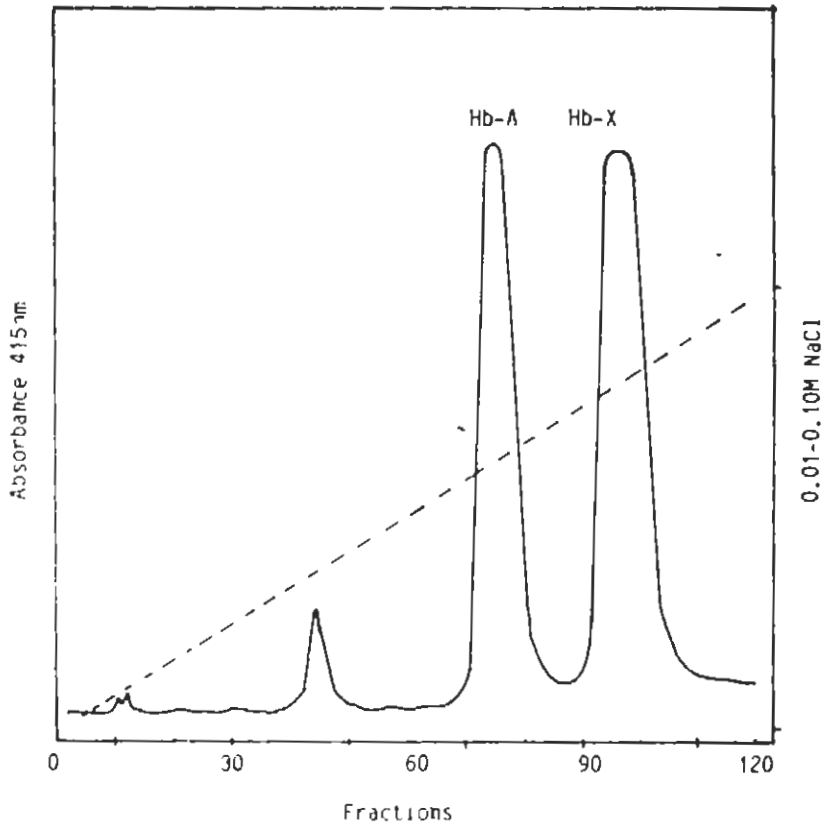


Table 3.1: Hematological data from the propositus and his family members.

| | (1) Father Carrier | (2) Son I Carrier | (3)Wife Normal | (4)Son II Normal | (5) Daughter Normal |
|---|---|---|---|---|---|
| Age year | 47 | 16 | 39 | 15 | 18 |
| RBC /pl | 5.35 | 5.54 | 4.61 | 4.67 | 4.57 |
| Hct (%/v/v) | 52.6 | 47.0 | 39.0 | 39.7 | 40.7 |
| Hb (g/dl) | 19.1 | 16.8 | 14.0 | 14.1 | 14.3 |
| MCV (fl) | 98.3 | 84.9 | 84.5 | 85.0 | 89.0 |
| MCV (pg) | 35.7 | 30.3 | 30.4 | 30.2 | 31.3 |

showed that both components are in about 1:1 ratio, which confirms the result of disc electrophoresis.

### 3.3.4: Chromatography on CM-Cellulose

Crude globin subjected to ion-exchange chromatography on CM-Cellulose column in the presence of 8M urea, resulted in the separation of abnormal β chain (Fig.3.4 .

### 3.3.5: Reversed phase HPLC of tryptic peptides

The fingerprinting of tryptic peptides by RP-HPLC on a column of LiChrosorb-RP2, resulted in the separation of an abnormal peptide. Elution profile of peptides is presented in Fig.3.5.

### 3.3.6: Amino acid analysis

Amino acid composition of the abnormal peptide isolated by reversed phase HPLC in presented in Table 3.2.

### 3.3.7: Amino acid sequence

Amino acid sequence of abnormal peptide determined in a gas-phase sequencer revealed that lysine at β144(HC1) is replaced with asparagine to which the normal Tp15 dipeptide tyrosyl-histidine is linked. These results confirmed it to be a case of Hb Andrew-Minneapolis.

| | 133 | 137 | 141 | 146 |
|---|---|---|---|---|
| $\beta^A$ Tp14 : | Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-**Lys**-Tyr-His | | | |
| $\beta^X$ Cp14-15: | | | Asn-Ala-Leu-Ala-His-**Asn**-Tyr-His | |
| $\beta^A$ Tp15 : | | | | Tyr-His |

Figure 3.4: Fractionation of the globin chains (Hb-Andrew-Minneapolis) on a CM-Cellulose (1.6x15cm) column. Buffer: 8M urea containing 0.025M sodium acetate and 0.2% mercaptoethanol pH5.8. .NaCl gradient: 0.02-0.08M.

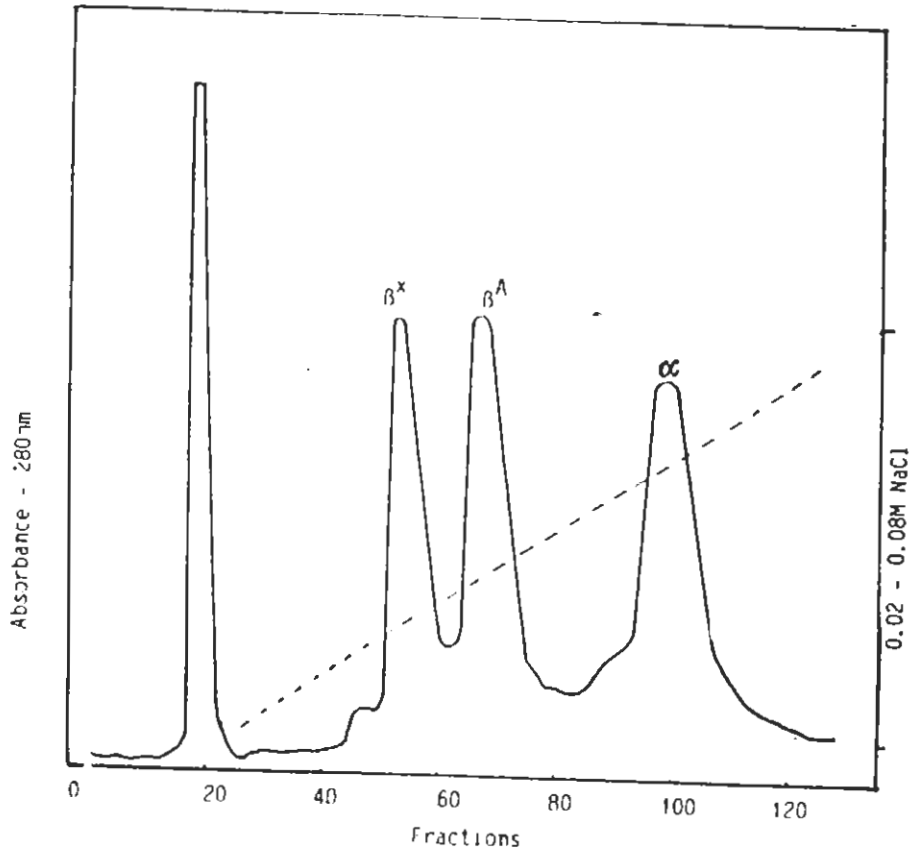


Figure 3.5: Separation profile of tryptic peptides from the oxidized $\beta^{X}$ chain of hemoglobin (Hb-Andrew-Minneapolis) on RP-HPLC. The abnormal peptide is shaded. Column: LiChrosorb-RP2; buffer 50mM ammonium acetate pH6.00; gradient 0-60% acetonitrile in 60min; flow rate 1ml/min.

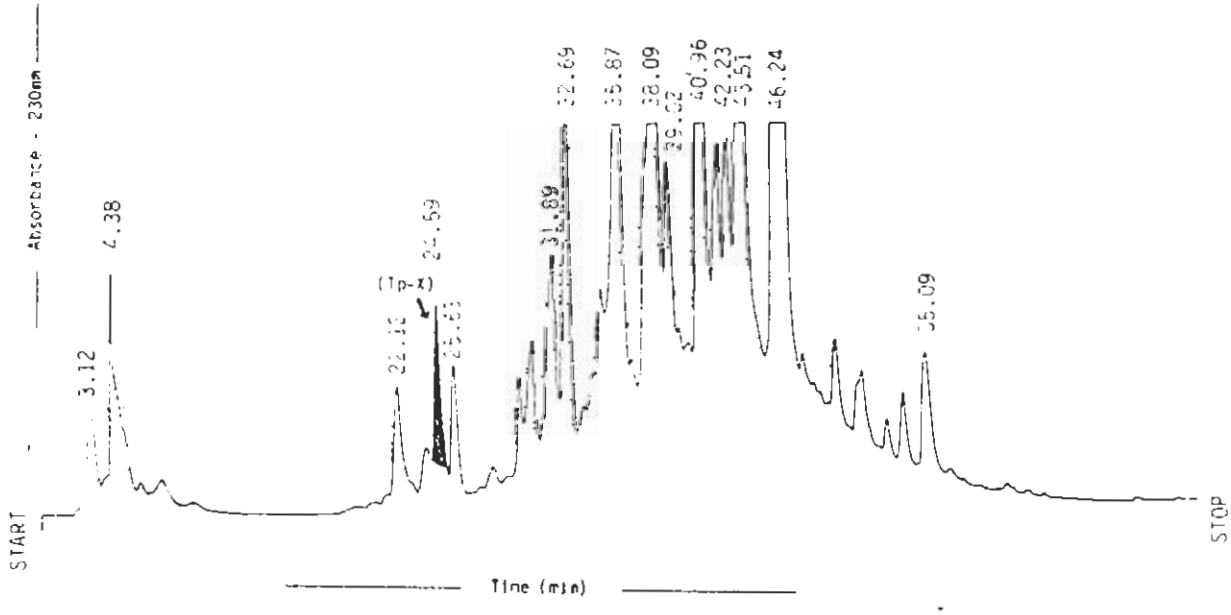


Table 3.2: Amino acid composition of the abnormal peptide. Numbers in parenthesis denote amino acid residue found during sequencing.

| Amino acid | $\beta^{X}$CTp14/15 | $\beta^{A}$Tp14 | $\beta^{A}$Tp15 |
|---|---|---|---|
| Asp | 1.15 (1) | (1) | - |
| Gly | - | (1) | - |
| Ala | 1.79 (2) | (4) | - |
| Val | - | (3) | - |
| Leu | 1.04 (1) | (1) | - |
| Tyr | 0.80 (1) | - | (1) |
| His | 1.90 (2) | (1) | (1) |
| Lys | - | (1) | - |
| Sum | 7 | 12 | 2 |

## 3.4: JAGUAR (Panthera onco) HEMOGLOBINS

### 3.4.1: Electrophoresis

Disc electrophoresis of hemolysate on a 10% polyacrylamide gel at pH8.3 revealed the presence of two hemoglobin components in 1:1 ratio (Fig.4.1a).

The native globin checked by polyacrylamide gel electrophoresis in the presence of dissociating agent 8M urea and Triton X-100 showed three chains namely α, βI, βII (Fig.4.1b).

### 3.4.2: Chromatography on CM-Cellulose

The separation of three globin chains α, βI, βII achieved by chromatography on the column of CM-Cellulose in the presence of 8M urea. The separation profile is presented in Fig.4.2. Triton electrophoresis of the separated chains showed the βII chain is being contaminated with the α-chain.

### 3.4.3: Reversed phase HPLC of globin chains

The native globin subjected to chromatography on the column of LiChrosorb-RP2, resulted in the resolution of three purified chains (Fig.4.3).

Figure 4.1: Electrophoretic pattern of jaguar hemolysate on polyacrylamide gel. a) Disc at pH8.3, b) Under dissociating condition, 8M urea and Triton-X100.

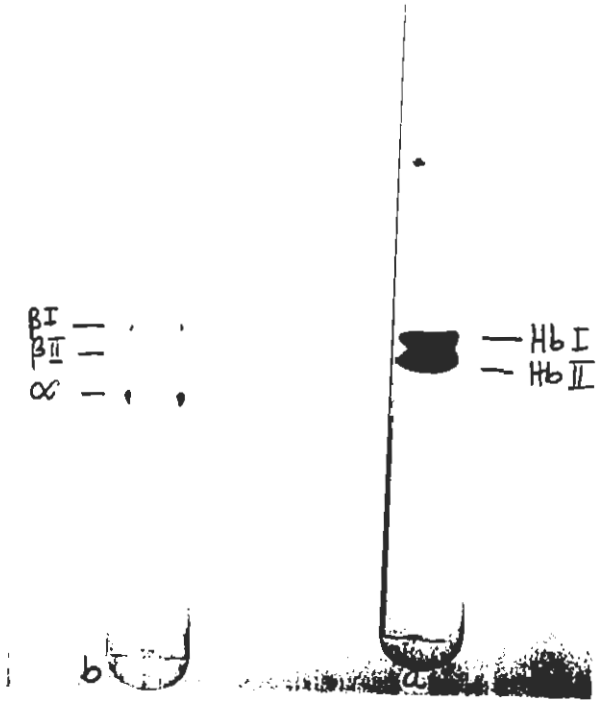


Figure 4.2: Separation profile of jaguar globin chains on CM-Cellulose column.

Buffer: 8M urea containing 0.025M sodium acetate, 0.2% mercaptoethanol pH5.8.

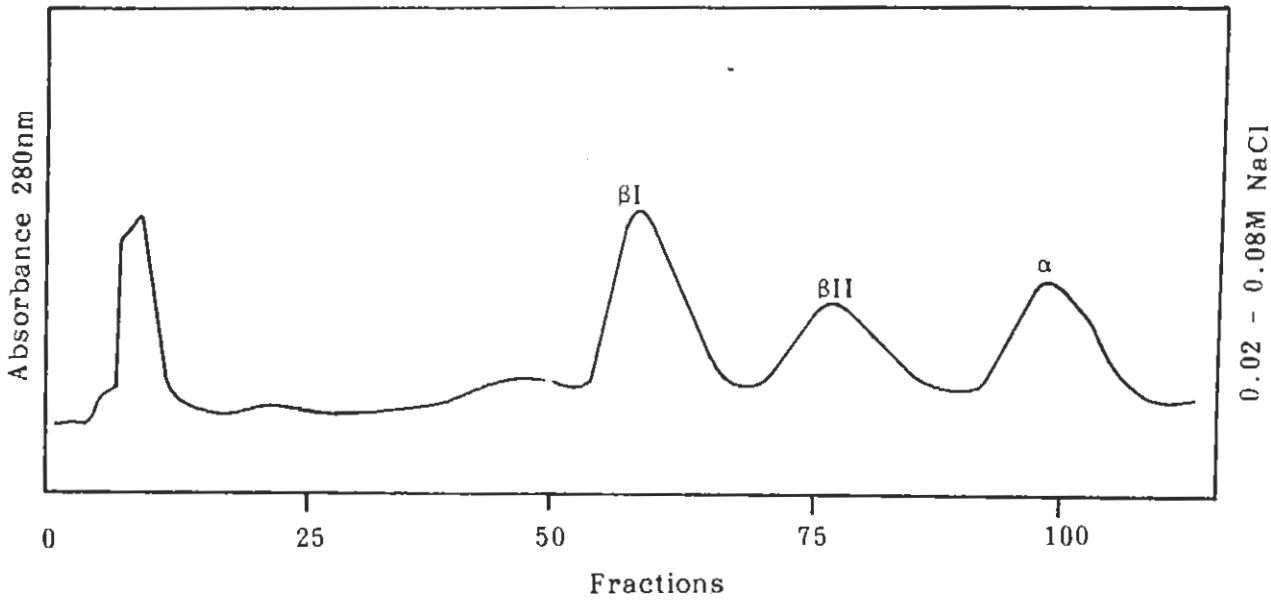
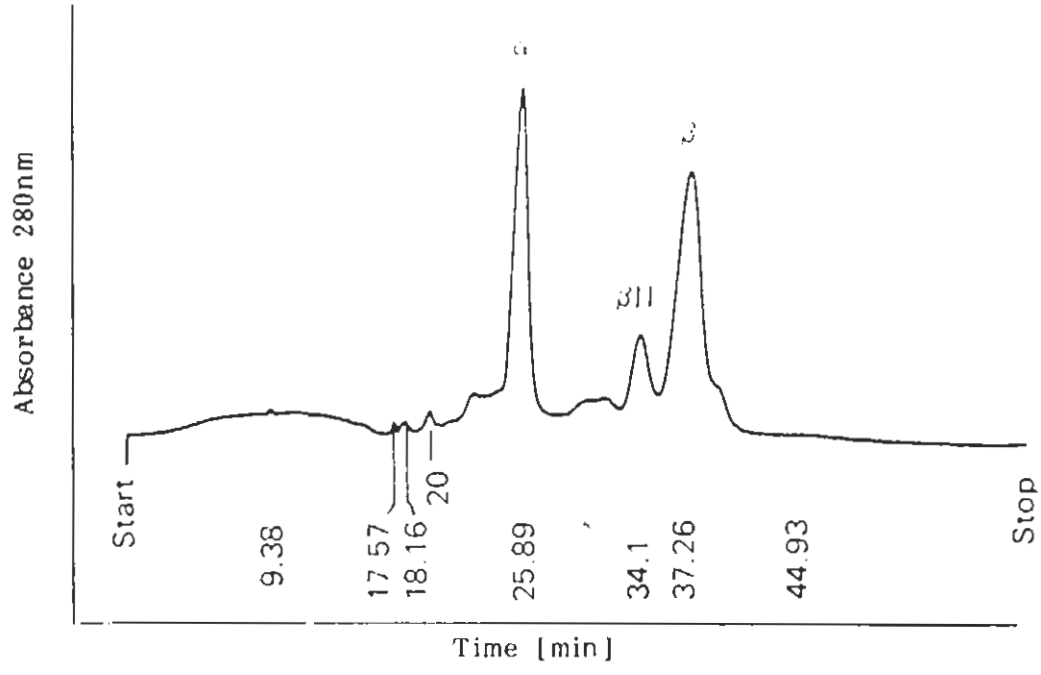


Figure 4.3 Elution pattern of jaguar globin chains on RP-HPLC column LiChrosorb RP2 (7µm).
Buffer: 12% formic acid/50mM ammonium acetate; gradient 0-35% acetonitrile in 2min, followed by 35-60% acetonitrile in 60min; flow rate 1ml/min; absorbance 280nm.



### 3.4.4: Prefractionation of tryptic peptides

The prefractionation of tryptic peptides on the column of Sephadex G-25(fine) gave some of the peptides in the pure form. The contaminated peptides obtained were purified by RP-HPLC (Fig.4.4).

### 3.4.5: Reversed phase HPLC of tryptic peptides

The prefractionated peptides obtained by gel filtration were rechromatographed by reversed phase HPLC on a column of LiChrosorb-RP2 led to pure peptides. Elution profile of some of the peptides of α and β chain purified on RP-HPLC is presented in Fig.4.5.

### 3.4.6: Amino acid analysis

Amino acid composition of tryptic peptides from α, βI and βII chains is presented in Table 4.1-4.3.

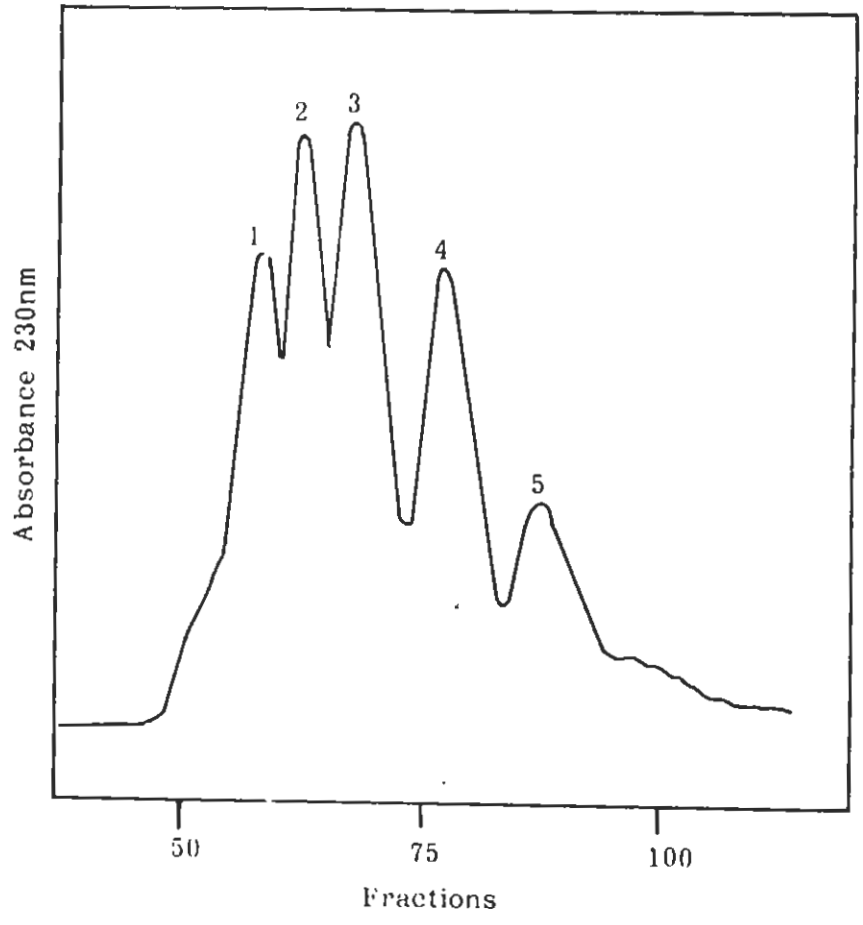
### 3.4.7: FAB-mass spectroscopy

By FAB-mass spectroscopy N-acetylserine was detected as a N-terminal amino acid residue of βI chain.

### 3.4.8: Amino acid sequence

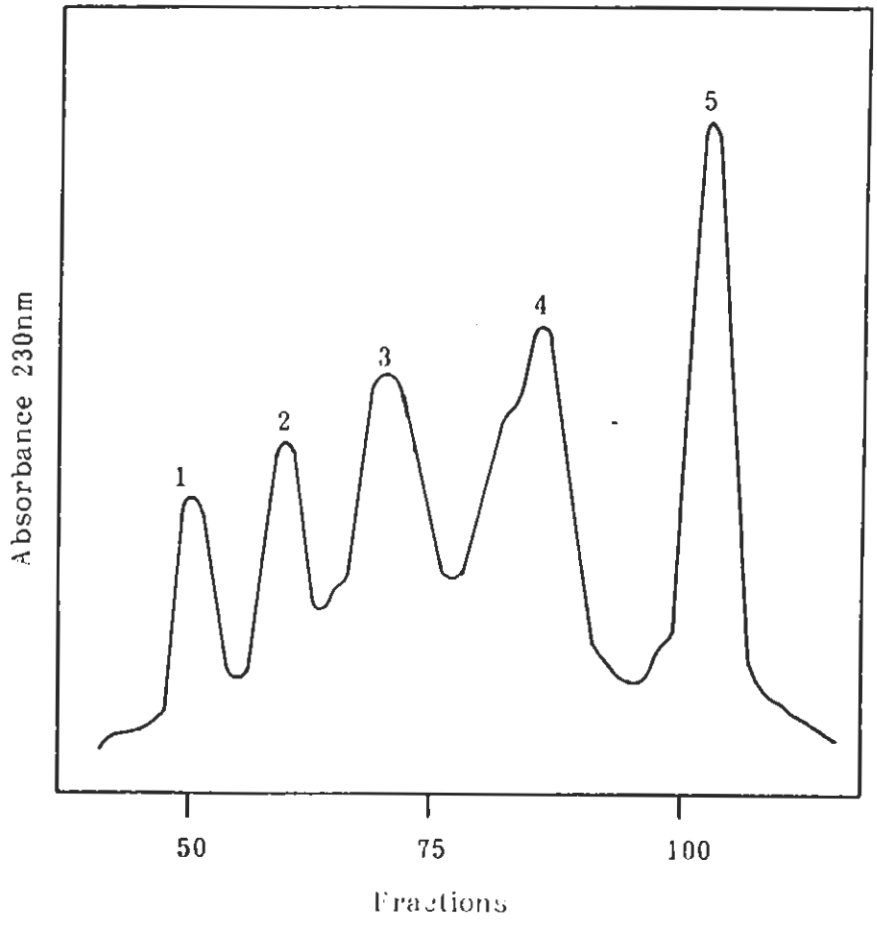
The complete amino acid sequence of α, βI and βII is presented in Fig.4.6. The sequence comparison with human Hb-A revealed 22(15.6%) substitution in α chain. 29(19.8%) in βI and 28(19.1%) in βII chain.

Figure 4.4a: Separation pattern of tryptic peptides from jaguar α-chain on column of Sephadex G25 (2.6x150cm), eluted with 0.1M acetic acid.




1: Tp12
2: Tp9b,12
3: Tp6-8
4: Tp4,9a,13
5: Tp1,2,3,5,10,11,14

Figure 4.4b: Separation pattern of tryptic peptides from jaguar β-chain on column of Sephadex G25 (2.6x150cm), eluted with 0.1M acetic acid.




1: Tp12-13
2: Tp10b,11,12-13chy
3: Tp3,5,10b,14
4: Tp1,2,4,6,7,8,9a,9b,10a,11
5: Tp15

Figure 4.5: Rechromatography of prefractioned tryptic peptides from Sephadex G25 on RP-HPLC Column: Lichrosorb RP2; buffer 50mM ammonium acetate pH6.00; gradient 0-60% acetonitrile in 60min; flow rate 1ml/min
a) Peptides from jaguar α-chain. b) Peptides from jaguar β-chain.

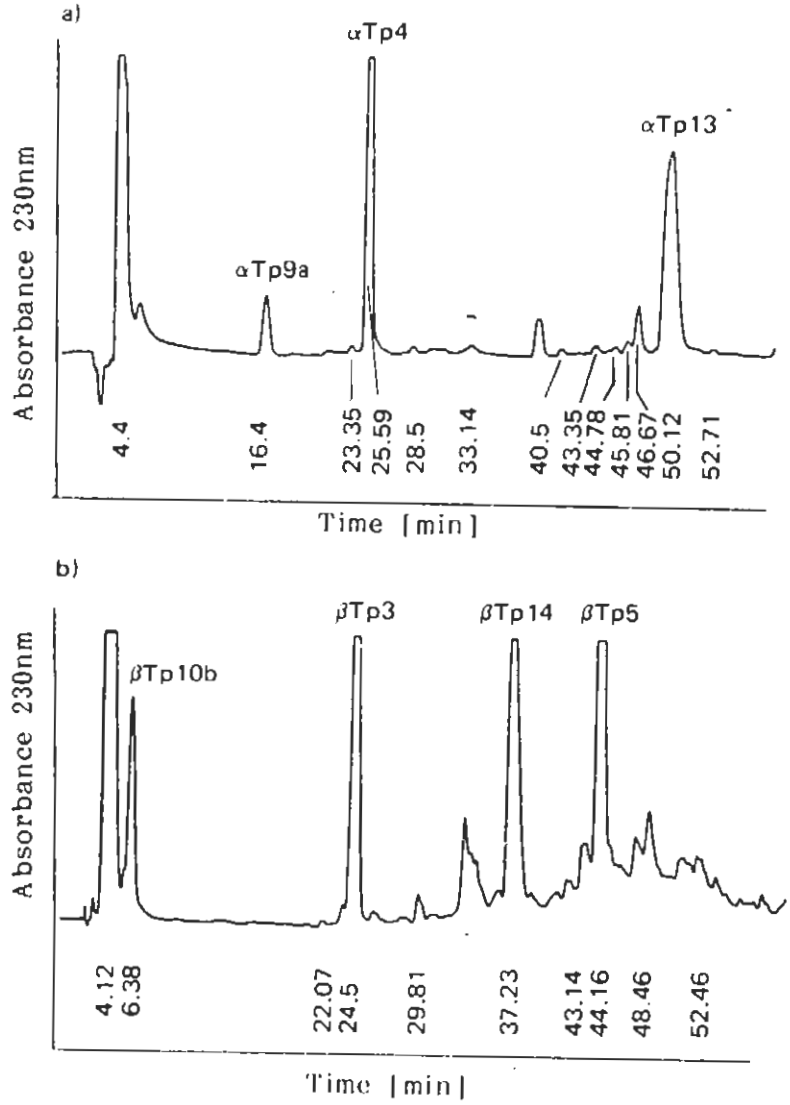


Table 4.1 Amino acid composition of peptides from α chain of Jaguar.

| Pos. | Tp1 1-7 | Tp2 8-11 | Tp3 12-16 | Tp4 17-31 | Tp5 32-40 | Tp6/7/8 41-61 | Tp9a 62-68 | Tp9b 69-90 | Tp10 91-92 | Tp11 93-99 | Tp12 100-127 | Tp13 128-139 | Tp14 140-141 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Asx | 1.08 | 1.91 | - | - | - | 1.14 | 0.92 | 4.68 | - | 1.95 | 1.00 | - | - |
| Thr | - | - | - | - | 2.79 | 0.89 | 0.93 | - | - | - | 2.05 | 1.93 | - |
| Ser | 1.80 | - | - | 0.89 | 1.10 | 1.80 | - | 1.85 | - | - | 1.60 | 2.92 | - |
| Glx | - | - | - | 3.13 | - | 3.19 | - | - | - | - | 2.05 | - | - |
| Pro | - | - | - | - | 1.23 | 0.89 | - | 1.06 | - | 1.16 | 2.02 | - | - |
| Gly | - | - | 1.02 | 2.85 | - | 2.03 | - | - | - | - | - | - | - |
| Ala | 1.00 | - | 0.97 | 3.08 | - | 2.17 | 1.75 | 4.05 | - | - | 2.77 | 1.04 | - |
| Cys* | - | - | 0.97 | - | 0.95 | - | - | - | - | - | 2.30(2) | - | - |
| Val | 0.96 | 0.98 | - | - | - | 0.98 | 0.91 | 1.04 | - | 1.91 | 1.75 | 2.05 | - |
| Ile | - | - | - | 0.96 | - | - | - | 0.90 | - | - | - | - | - |
| Leu | 1.08 | - | - | 1.04 | - | 1.10 | 1.45 | 4.20 | 1.02 | - | 4.70 | 1.05 | - |
| Tyr | - | - | - | 0.73 | - | 0.83 | - | 0.83 | - | - | - | - | 0.76 |
| Phe | - | - | - | - | 1.91 | 1.96 | - | - | - | 0.98 | 2.30(2) | 1.97 | - |
| His | - | - | - | 1.26 | - | 3.09 | - | 2.31 | - | - | 4.11 | - | - |
| Trp | - | - | 0.81 | - | - | - | - | - | - | - | - | - | - |
| Lys | 1.06 | 1.10 | 1.03 | - | 1.00 | 0.98 | 1.02 | 1.02 | - | 0.99 | 0.88 | 0.98 | - |
| Arg | - | - | - | 1.01 | - | - | - | - | 1.2 | - | - | - | 0.81 |
| Sum | 7 | 4 | 5 | 15 | 9 | 21 | 7 | 22 | 2 | 7 | 28 | 12 | 2 |

* Determined after performic acid oxidation.
Values in brackets are taken from sequence data.

Table 4.2 Amino acid composition of peptides from β I chain of Jaguar.

| Pos. | Tp1 1-8 | Tp2 9-17 | Tp3 18-30 | Tp4 31-40 | Tp5 41-59 | Tp6 60-61 | Tp7 62-65 | Tp8 66 | Tp9a 67-76 | Tp9b 77 82 | Tp10a 83-87 | Tp10b 88-95 | Tp11 96-104 | Tp12/13 105-132 | Tp14 133-144 | Tp15 145-146 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Asx | - | 1.28 | 2.04 | - | 3.04 | - | - | - | 2.07 | 3.07 | - | 1.05 | 2.03 | 2.10 | - | - |
| Thr | - | - | - | 1.00 | - | - | - | - | - | - | - | - | - | - | - | - |
| Ser | 1.82 | 1.00 | - | - | 3.68 | - | - | - | 1.77 | - | - | 0.89 | - | - | 0.98 | - |
| Glx | 2.20 | - | 2.16 | 1.03 | 1.18 | - | - | - | - | - | - | 1.07 | 1.14 | 4.38(4) | - | - |
| Pro | - | - | - | 0.90 | - | - | - | - | - | - | - | - | 0.95 | 0.75 | - | - |
| Gly | - | 1.71 | 2.90 | - | 1.13 | - | 0.99 | - | 1.13 | - | 0.87 | - | - | 2.12 | 1.18 | - |
| Ala | 0.96 | - | 1.05 | - | 3.15 | - | 1.00 | - | - | - | 2.06 | - | - | 3.03 | 4.14 | - |
| Cys* | - | - | - | - | - | - | - | - | - | - | - | 1.02 | - | 1.04 | - | - |
| Val | - | 1.17 | 2.88 | 1.53(2) | - | 1.09 | - | - | 1.00 | - | - | - | 0.96 | 3.89 | 2.64 | - |
| Met* | - | - | - | - | 1.01 | - | - | - | - | - | - | - | - | - | - | - |
| Ile | - | - | - | - | 0.81 | - | - | - | - | 0.95 | - | | - | - | - | - |
| Leu | 1.02 | 2.07 | 1.00 | 2.13 | 1.03 | - | - | - | 2.05 | 0.98 | - | 1.99 | 1.05 | 3.99 | 1.15 | - |
| Tyr | - | - | - | 0.81 | - | - | - | - | - | - | - | - | - | - | - | 0.87 |
| Phe | 0.96 | - | - | - | 2.96 | - | - | - | 0.98 | - | 1.02 | - | 0.99 | 2.89 | - | - |
| His | - | - | - | - | - | - | 0.93 | - | - | - | - | 1.00 | 0.91 | 2.95 | 1.05 | 1.12 |
| Trp | - | 0.73 | - | 0.87 | - | - | - | - | - | - | - | - | - | - | - | - |
| Lys | 1.01 | 0.85 | - | - | 0.99 | 0.92 | 1.05 | 1.05 | 1.00 | 1.00 | 0.86 | 0.96 | - | 0.95 | - | - |
| Arg | - | - | 0.94 | 1.23 | - | - | - | - | - | - | - | - | 0.93 | - | 1.04 | - |
| Sum | 8 | 9 | 13 | 10 | 19 | 2 | 4 | 1 | 10 | 6 | 5 | 8 | 9 | 28 | 12 | 2 |

* Determined after performic acid oxidation.
Values in brackets are taken from sequence data.

Table 4.3 Amino acid composition of peptides from β II chain of Jaguar.

| Pos. | Tp1 1-8 | Tp2 9-17 | Tp3 18-30 | Tp4 31-40 | Tp5 41-59 | Tp6 60-61 | Tp7 62-65 | Tp8 66 | Tp9a 67-76 | Tp9b 77-82 | Tp10a 83-87 | Tp10b 88-95 | Tp11 96-104 | Tp12/13 105-132 | Tp14 133-144 | Tp15 145-146 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Asx | - | 1.26 | 1.94 | - | 3.19 | - | - | - | 1.92 | 2.66 | - | 1.02 | 1.99 | 2.09 | - | - |
| Thr | - | - | - | 0.94 | - | - | - | - | - | - | - | - | - | - | - | - |
| ser | 0.99 | 1.01 | - | - | 3.67 | - | - | - | 1.54(2) | - | - | 0.91 | - | - | 1.08 | - |
| Glx | 1.94 | - | 2.10 | 1.03 | 1.04 | - | - | - | - | - | - | 1.14 | 1.05 | 3.93 | - | - |
| Pro | - | - | - | 0.85 | - | - | - | - | - | - | - | - | 0.78 | 1.06 | - | - |
| Gly | 1.04 | 1.89 | 2.73 | - | 1.24 | - | 1.04 | - | 1.33(1) | - | 0.98 | - | - | 2.14 | 1.19 | - |
| Ala | 0.95 | - | 1.10 | - | 2.98 | - | 1.15 | - | - | - | 1.92 | - | - | 2.96 | 3.73 | - |
| Cys* | - | - | - | - | - | - | - | - | - | - | - | 0.85 | - | 0.96 | - | - |
| Val | - | 0.91 | 2.94 | 1.62 | - | 1.04 | - | - | 1.02 | - | - | - | 1.05 | 4.01 | 2.58 | - |
| Met* | - | - | - | - | 0.70 | - | - | - | - | - | - | - | - | - | - | - |
| Ile | - | - | - | - | 1.00 | - | - | - | - | 1.11 | - | - | - | - | - | - |
| Leu | 1.09 | 1.79 | 1.13 | 2.14 | 1.10 | - | - | - | 2.14 | 1.16 | - | 2.03 | 1.08 | 4.11 | 1.28 | - |
| Tyr | - | - | - | 0.90 | - | - | - | - | - | - | - | - | - | - | - | 0.87 |
| Phe | 0.96 | - | - | - | 2.89 | - | - | - | 1.03 | - | 1.01 | - | 0.96 | 2.75 | - | - |
| His | - | - | - | - | - | - | 1.05 | - | - | - | - | 1.01 | 1.02 | 2.96 | 1.12 | 1.12 |
| Trp | - | 0.82 | - | 0.79 | - | - | - | - | - | - | - | - | - | - | - | - |
| Lys | 0.89 | 1.18 | - | - | 1.16 | 0.98 | 0.80 | 1.01 | 0.98 | 1.05 | 1.00 | 1.00 | - | 0.98 | 1.15 | - |
| Arg | - | - | 1.02 | 1.10 | - | - | - | - | - | - | - | - | 0.92 | - | - | - |
| Sum | 8 | 9 | 13 | 10 | 19 | 2 | 4 | 1 | 10 | 6 | 5 | 8 | 9 | 28 | 12 | 2 |

* Determined after performic acid oxidation.
Values in brackets are taken from sequence data.

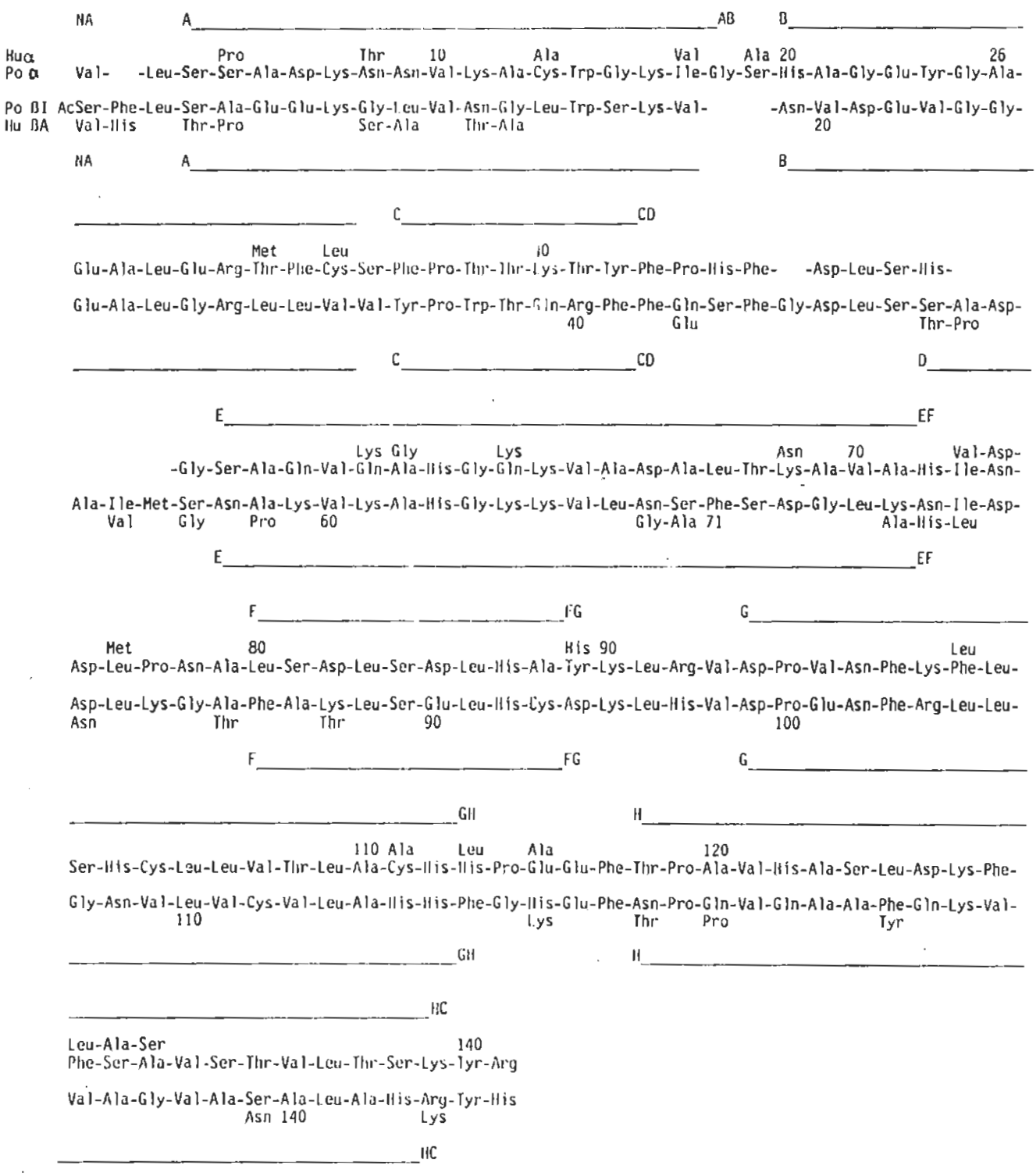



Fig.4.6:

Amino acid sequence of Jaguar (Po) globin chains in aligment with the corresponding chain of human (Hu). In case of human hemoglobin only the exchanges are given. The Hb-II differs from the Hb-I at the following positions.
βI/βII: βNA1 Ac-Ser/Gly, βIIC1 Arg/Lys.

Between two β chains only two differences were found at βI/βII: β1(NA1) Ac-Ser/Gly, and β144(HC1) Arg/Lys.

The exchanges which were distributed over the entire length of the hemoglobin molecule resulted in the alteration of four α1β1 contact points: α34(B15) Leu/Cys, α111(G18) Ala/Cys, β123(H1) Thr/Asn, β125(H3) Pro/Gln. One α1β2 contact point at β43(CD2) Glu/Gln and one heme contact point at β70(E14) Ala/Ser. Among the 2,3-diphosphoglycerate binding sites two exchanges were observed at β1 (NA1) Val/Ac-Ser and β2 (NA2) His/Phe.

## 3.5: NORTH PERSIAN LEOPARD (Panthera pardus sexicolor) HEMOGLOBINS

### 3.5.1: Electrophoresis

Hemoglobin of North Persian Leopard consists of two components as verified by polyacrylamide gel disc electrophoresis (Fig.5.1a). The major component accounting 80-90% of the total hemoglobin and the minor component making up for the rest.

Electrophoresis of crude globin on polyacrylamide gel under dissociating condition in the presence of 8M urea and Triton X-100 showed three globin chains α, βI and βII (Fig.5.1b).

### 3.5.2: Chromatography on CM-Cellulose

The separation of globin chains was achieved by chromatography on the column of CM-Cellulose. It confirmed the result of Triton electrophoresis. The separation profile presented in Fig.5.2.

### 3.5.3: Reversed phase HPLC of hemoglobin

The hemolysate subjected on a reversed phase HPLC, column of Nucleosil-C4, resulted in the separation of three globin chains. The separation profile (Fig.5.3) shows the elution of heme followed by α, βII and βI chains.

Figure 5.1: Electrophoretic pattern of leopard hemolysate on polyacrylamide gel.
a) Disc at pH8.3. b) Under dissociating condition, 8M urea and Triton X-100.

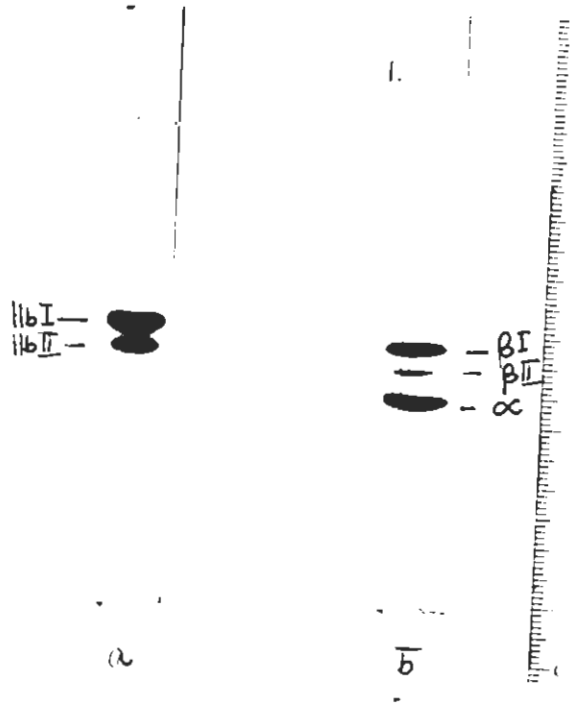


Figure 5.2: Separation of the globin chains of leopard on CM-Cellulose column (1.6x150cm).

Buffer: 8M urea containing 0.025 sodium acetate and 0.2% mercaptoethanol pH5.8; NaCl gradient 0.02-0.08M.

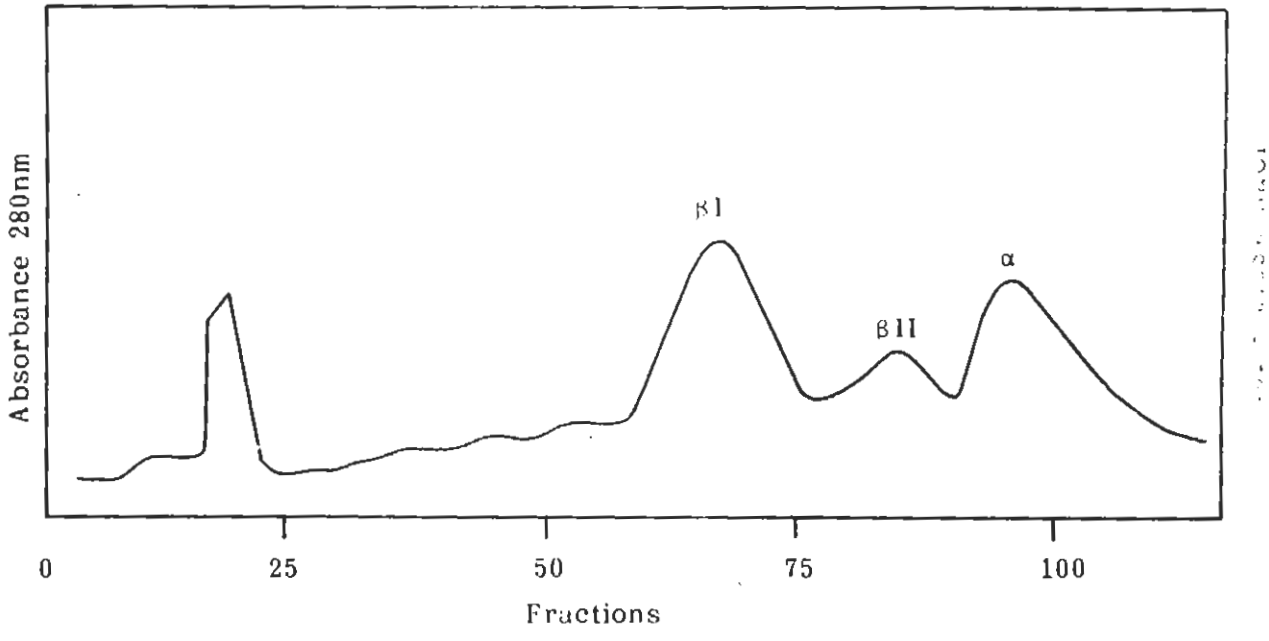
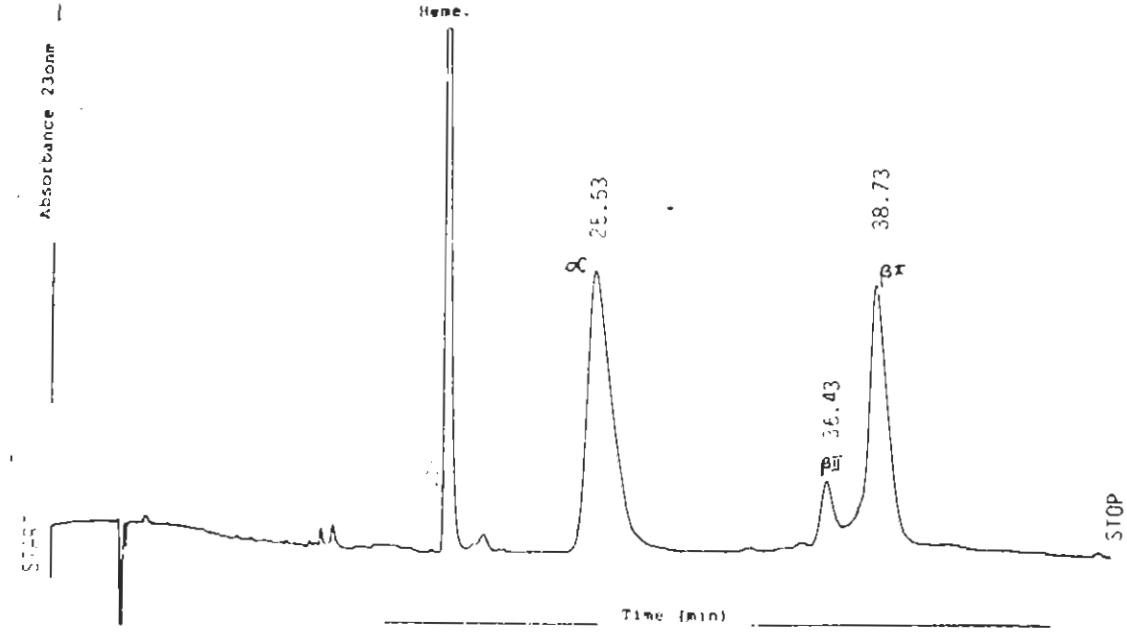


Figure 5.3: Fractionation of the heme and globin chains of leopard on RP-HPLC.
Column: Nucleosil-C4 (7µM); buffer 0.1% TFA; gradient 0-35% acetonitrile in 2min, followed by 35-60% in 60min; flow rate 1ml/min; absorbance 230nm.



### 3.5.4: Separation of tryptic peptides by RP-HPLC

The globin chain digested with TPCK-trypsin were subjected directly to a column of LiChrosorb-RP2 resulted in the separation of tryptic peptides. The impure peptides obtained were rechromatographed on Vydac-C18 column led to the isolation of pure peptides. The elution profile of tryptic peptides from the α and β chains is shown in Fig.5.4-5.5.

### 3.5.5: Amino acid analysis

Amino acid composition of the tryptic peptides from α, βI and βII chains is presented in Table 5.1-5.3.

### 3.5.6: Amino acid sequence

The complete primary structure of the hemoglobins was established to some extent by sequencing the N-terminal part of the native chain upto 42 residues, but mainly by sequencing the tryptic peptides.

The amino acid sequence of the globin chains is presented in Fig.5.6. The sequence aligned with that of human Hb-A showed 22 amino acid exchanges in the α chain, β29 in βI and 28 in βII chains. Within the two β chains only two exchanges have been located at βI/βII: β1(NA1) Ac-Ser/Gly and β144(HC1) Arg/Lys. Among the functionally important positions, eight substitutions were observed; four in α1β1, one in the

Figure 5.4: Separation pattern of tryptic peptides from the oxidized α-chain of leopard. Column: LiChrosorb-RP2; buffer 50mM ammonium acetate pH6.00; gradient 0-60% acetonitrile in 70min; flow rate 1ml/min.

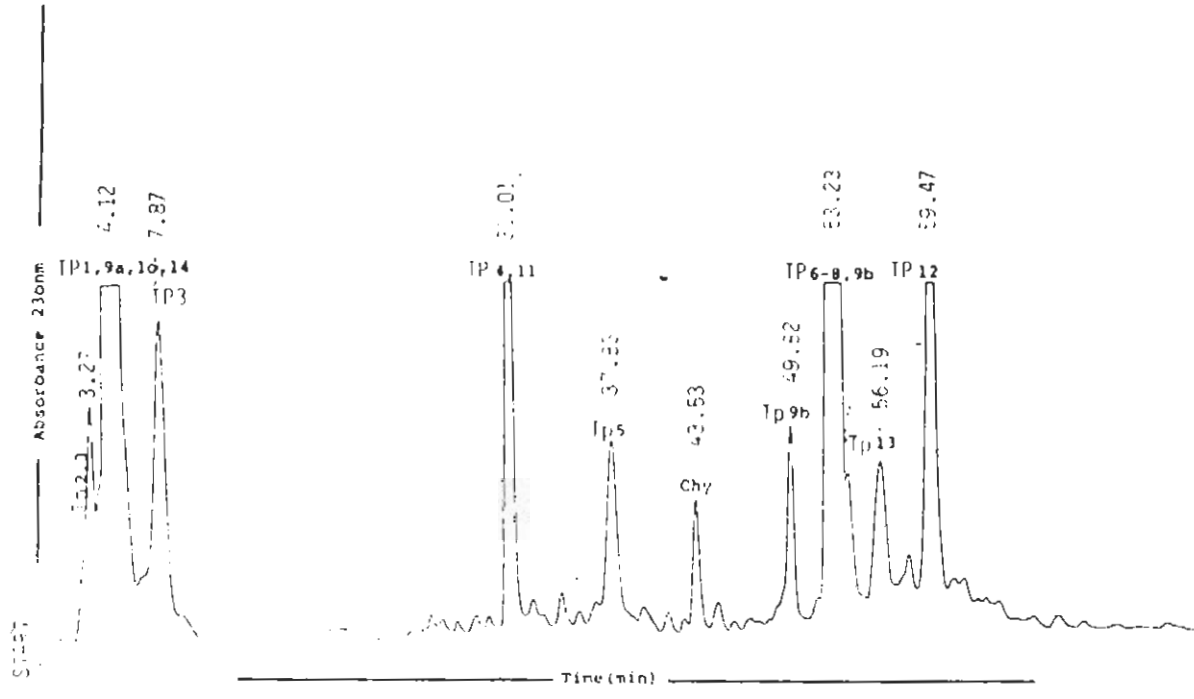


Figure 5.4 : RP-HPLC pattern of tryptic peptides from oxidized β-chain of leopard.
Column: LiChrosorb-RP2; buffer 50mM ammonium acetate pH6.0; gradient 0-60% acetonitrile in 70min; flow rate 1ml/min.

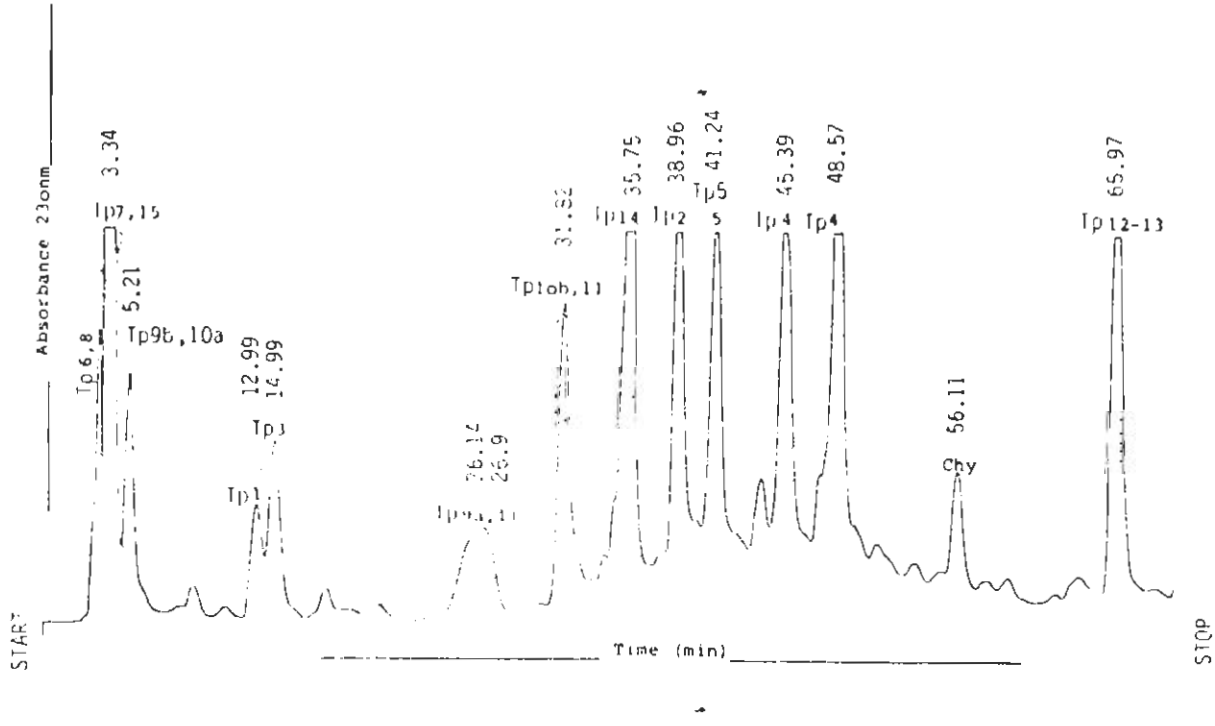


Table 5.1 Amino acid composition of peptides from α chain of Leopard.

| Pos. | Tp1 1-7 | Tp2 8-11 | Tp3 12-16 | Tp4 17-31 | Tp5 32-40 | Tp6/7/8 41-61 | Tp9a 62-68 | Tp9b 69-90 | Tp10 91-92 | Tp11 93-99 | Tp12 100-127 | Tp13 128-139 | Tp14 140-141 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Asx | 1.01 | 1.81 | - | - | - | 1.03 | 1.02 | 4.68 | - | 1.85 | 1.21 | - | - |
| Thr | - | - | - | - | 2.79 | 0.89 | 1.06 | - | - | - | 1.90 | 1.92 | - |
| Ser | 1.82 | - | - | 0.89 | 1.00 | 1.87 | - | 1.77 | - | - | 2.12 | 2.72 | - |
| Glx | - | - | - | 3.23 | - | 3.19 | - | - | - | - | 2.24 | - | - |
| Pro | - | - | - | - | 1.13 | 0.89 | - | 1.03 | - | 1.21 | 2.19 | - | - |
| Gly | - | - | 1.02 | 2.79 | - | 1.93 | - | - | - | - | - | - | - |
| Ala | 1.00 | - | 0.97 | 3.08 | - | 1.97 | 1.85 | 3.85 | - | - | 2.85 | 1.25 | - |
| Cys* | - | - | 0.97 | - | 0.95 | - | - | - | - | - | 2.10 | - | - |
| Val | 0.86 | 0.88 | - | - | - | 0.98 | 0.91 | 1.14 | - | 1.80 | 1.86 | 2.03 | - |
| Ile | - | - | - | 0.96 | - | - | - | 0.90 | - | | - | - | - |
| Leu | 1.08 | - | - | 1.14 | - | 1.10 | 1.03 | 3.94 | 0.82 | - | 4.62 | 1.20 | - |
| Tyr | - | - | - | 0.81 | - | 0.80 | - | 0.79 | - | - | - | - | 0.91 |
| Phe | - | - | - | - | 1.91 | 1.89 | - | - | - | 0.96 | 2.15 | 1.91 | - |
| His | - | - | - | 0.98 | - | 2.89 | - | 2.21 | - | - | 3.52(4) | - | - |
| Trp | - | - | 0.78 | - | - | - | - | - | - | - | - | - | - |
| Lys | 1.06 | 0.90 | 1.13 | - | 1.13 | 1.07 | 1.05 | 1.09 | - | 1.01 | 1.12 | 1.07 | - |
| Arg | - | - | - | 1.04 | - | - | - | - | 0.93 | - | - | - | 1.01 |
| Sum | 7 | 4 | 5 | 15 | 9 | 21 | 7 | 22 | 2 | 7 | 28 | 12 | 2 |

* Determined after performic acid oxidation.
Values in brackets are taken from sequence data.

Table 5.2 Amino acid composition of peptides from β I chain of Leopard.

| Pos. | Tp1 1-8 | Tp2 9-17 | Tp3 18-30 | Tp4 31-40 | Tp5 41-59 | Tp6 60-61 | Tp7 62-65 | Tp8 66 | Tp9a 67-76 | Tp9b 77 82 | Tp10a 83-87 | Tp10b 88-95 | Tp11 96-104 | Tp12/13 105-132 | Tp14 133-144 | Tp15 145-146 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Asx | - | 1.21 | 1.89 | - | 2.90 | - | - | - | 1.89 | 2.96 | - | 0.87 | 1.94 | 2.15 | - | - |
| Thr | - | - | - | 1.00 | - | - | - | - | - | - | - | - | - | - | - | - |
| Ser | 1.66 | 0.80 | - | - | 3.68 | - | - | - | 2.10 | - | - | 0.79 | - | - | 1.13 | - |
| Glx | 1.86 | - | 2.12 | 0.97 | 0.86 | - | - | - | - | - | - | 1.07 | 1.04 | 4.21 | - | - |
| Pro | - | - | - | 0.86 | - | - | - | - | - | - | - | - | 0.89 | 1.37(1) | - | - |
| Gly | - | 1.81 | 2.50(3) | - | 1.29 | - | 0.99 | - | 0.87 | - | 0.96 | - | - | 1.89 | 1.14 | - |
| Ala | 0.92 | - | 1.05 | - | 3.17 | - | 1.02 | - | - | - | 1.83 | - | - | 3.12 | 4.06 | - |
| Cys* | - | - | - | - | - | - | - | - | - | - | - | 0.93 | - | 0.96 | - | - |
| Val | - | 1.01 | 2.88 | 1.59(2) | - | 0.99 | - | - | 0.79 | - | - | - | 0.93 | 3.69 | 2.30(3) | - |
| Met* | - | - | - | - | 0.98 | - | - | - | - | - | - | - | - | - | - | - |
| Ile | - | - | - | - | 0.85 | - | - | - | - | 0.89 | - | - | - | - | - | - |
| Leu | 0.97 | 2.01 | 1.00 | 2.13 | 1.00 | - | - | - | 2.04 | 1.03 | - | 1.86 | 1.12 | 3.81 | 1.20 | - |
| Tyr | - | - | - | 0.85 | - | - | - | - | - | - | - | - | - | - | - | 0.83 |
| Phe | 0.96 | - | - | - | 3.16 | - | - | - | 0.98 | - | 0.88 | - | 1.06 | 3.05 | - | - |
| His | - | - | - | - | - | - | 1.03 | - | - | - | - | 0.96 | 0.91 | 3.01 | 1.05 | 0.98 |
| Trp | - | 0.80 | - | 0.79 | - | - | - | - | - | - | - | - | - | - | - | - |
| Lys | 1.00 | 0.85 | - | - | 0.99 | 0.87 | 1.02 | 1.02 | 0.95 | 1.00 | 0.89 | 0.97 | - | 0.95 | - | - |
| Arg | - | - | 0.98 | 1.13 | - | - | - | - | - | - | - | - | 1.04 | - | 1.14 | - |
| Sum | 8 | 9 | 13 | 10 | 19 | 2 | 4 | 1 | 10 | 6 | 5 | 8 | 9 | 28 | 12 | 2 |

* Determined after performic acid oxidation.
Values in brackets are taken from sequence data.

Table 5.3 Amino acid composition or peptides from β II chain of Leopard.

| Pos. | Tp1 1-8 | Tp2 9-17 | Tp3 18-30 | Tp4 31-40 | Tp5 41-59 | Tp6 60-61 | Tp7 62-65 | Tp8 66 | Tp9a 67-76 | Tp9b 77-82 | Tp10a 83-87 | Tp10b 88-95 | Tp11 96-104 | Tp12/13 105-132 | Tp14 133-144 | Tp15 145-146 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Asx | - | 0.98 | 1.88 | - | 3.00 | - | - | - | 2.12 | 2.81 | - | 1.18 | 2.13 | 2.10 | - | - |
| Thr | - | - | - | 1.00 | - | - | - | - | - | - | - | - | - | - | - | - |
| ser | 0.87 | 0.86 | - | - | 3.67 | - | - | - | 1.84 | - | - | 0.95 | - | - | 1.04 | - |
| Glx | 1.78 | - | 2.17 | 0.98 | 0.87 | - | - | - | - | - | - | 1.00 | 0.89 | 3.97 | - | - |
| Pro | - | - | - | 0.87 | - | - | - | - | - | - | - | - | 0.88 | 1.04 | - | - |
| Gly | 0.88 | 1.87 | 3.04 | - | 1.00 | - | 0.97 | - | 1.12 | - | 0.92 | - | - | 2.27 | 0.95 | - |
| Ala | 0.82 | - | 1.30(1) | - | 2.72 | - | 1.05 | - | - | - | 1.71 | - | - | 2.95 | 4.03 | - |
| Cys* | - | - | - | - | - | - | - | - | - | - | - | 0.85 | - | 0.83 | - | - |
| Val | - | 0.98 | 2.68 | 1.62(2) | - | 1.04 | - | - | 0.93 | - | - | - | 0.97 | 3.87 | 2.41(3) | - |
| Met* | - | - | - | - | 0.86 | - | - | - | - | - | - | - | - | - | - | - |
| Ile | - | - | - | - | 0.77 | - | - | - | - | 1.03 | - | - | - | - | - | - |
| Leu | 1.01 | 1.89 | 1.04 | 2.10 | 1.20 | - | - | - | 2.03 | 1.06 | - | 1.96 | 1.08 | 4.01 | 1.20 | - |
| Tyr | - | - | - | 0.90 | - | - | - | - | - | - | - | - | - | - | - | 1.01 |
| Phe | 0.96 | - | - | - | 2.86 | - | - | - | 1.01 | - | 0.86 | - | 0.98 | 3.04 | - | - |
| His | - | - | - | - | - | - | 1.01 | - | - | - | - | 1.01 | 0.99 | 3.09 | 0.96 | 1.14 |
| Trp | - | 0.76 | - | 0.89 | - | - | - | - | - | - | - | - | - | - | - | - |
| Lys | 0.95 | 1.11 | - | - | 1.10 | 0.98 | 1.01 | 1.01 | 1.07 | 1.00 | 1.00 | 1.13 | - | 0.93 | 1.00 | - |
| Arg | - | - | 1.01 | 0.89 | - | - | - | - | - | - | - | - | 0.98 | - | - | - |
| Sum | 8 | 9 | 13 | 10 | 19 | 2 | 4 | 1 | 10 | 6 | 5 | 8 | 9 | 28 | 12 | 2 |

* Determined after performic acid oxidation.
Values in brackets are taken from sequence data.

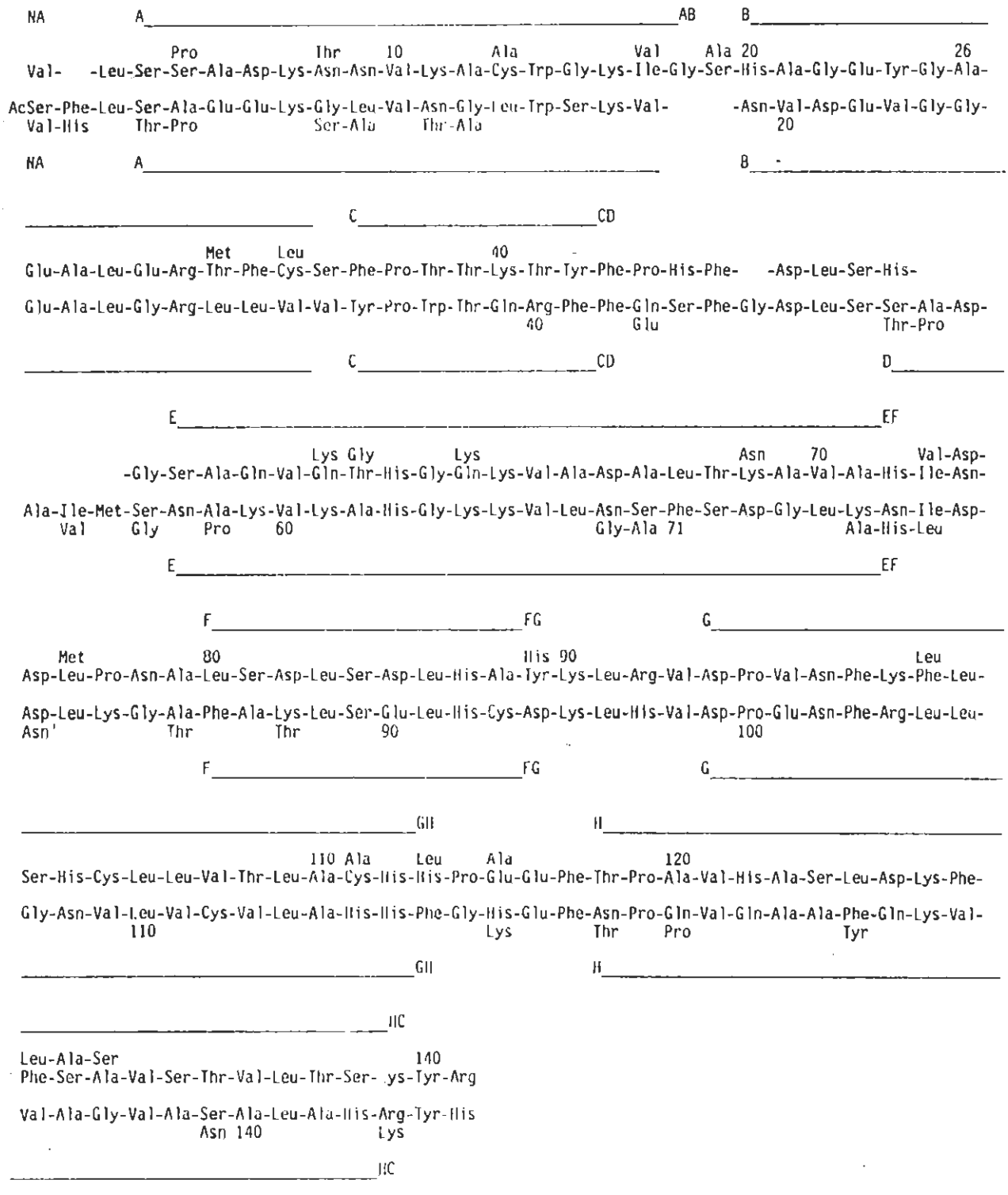



Fig.5.6
Amino acid sequence of Leopard (Pp) globin chains in alignment with the corresponding chain of human (Hu). In case of human hemoglobin only the exchanges are given. The Hb-II differs from the Hb-I at the following positions.
β1/β11: βNA1 Ac-Ser/Gly, βIIC1 Arg/Lys.

β1, βII and one in the heme contact point. Two exchanges were located at binding sites for 2,3-diphosphoglycerate, β1(NA1) and β2(NA2) with Ac-Ser and Phe respectively.

# 4: DISCUSSION

## 4.1: HEMOGLOBIN E β-THALASSEMIA

Hemoglobin E $\alpha_2 \beta_2^{26Glu \rightarrow Lys}$ is a slow moving variant of hemoglobin discovered by Itano et al. 1954 [116] and characterized by Hunt et al.1961 [117]. This variant is found with high frequency in Southeast Asia, especially in Thailand [118].

Cases of Hb-E, Hb-E/β-thalassemia have been reported from Rawalpindi and Karachi [119,120], on the basis of electrophoretic mobility. In the present study we have characterized the amino acid sequence of abnormal peptides by using liquid phase sequencer.

The propositus a 15 year old boy, suffering with severe clinical problem including continued jaundice, was getting periodic blood transfusion for the last six years. Hematological, biochemical and radiological examination of blood suggested a β-thalassemia abnormality.

Electrophoresis of its hemolysate on polyacrylamide disc and cellulose acetate membrane showed absence of Hb-A, a band at the position of Hb-$A_2$ and an elevated Hb-F (about 45%). This was confirmed by Triton electrophoresis and also by reversed phase HPLC. The elution profile shown in Fig.1.4 revealed an abnormal β chain followed by α and absence of $\beta^A$ chain. Small peaks in pre α region showed that the abnormal component to be contaminated with Hb-$A_2$. Separation of Hb-$A_2$ from Hb-E was not achieved by either electrophoresis or

ion-exchange chromatography of the hemolysate. Slightly elevated value for Hb-$A_2$ also suggests it to be a case of β-thalassemia.

The abnormal peptide isolated by RP-HPLC analysed for its amino acid composition followed by amino acid sequence in liquid-phase sequencer. The sequence established revealed glutamyl residue in peptide Tp3a β26 (B8) replaced with lysyl residue. These results confirmed it to be a case of Hb-E in association with β thalassemia. The prevalence of this variant is the third most common variant of hemoglobin after Hb-S $\alpha_2\beta_2^{6Glu \rightarrow Val}$ [121], and Hb-C $\alpha_2\beta_2^{6Glu \rightarrow Lys}$ [122]. Hemoglobin E produces no clinical symptom in heterozygous condition. In homozygous condition, hemoglobin level may be somewhat lower than normal and a slight hemolytic anemia with moderate spleenic enlargement is observed. The association of Hb-E with β thalassemia results in very severe disorders i.e. severe anemia is hypochromic and microcytic. The severe condition of the subject under study is also due to this hemoglobinopathy.

## 4.2: HEMOGLOBIN KARACHI

Hemoglobin Karachi $\alpha_2\beta_2^{5Ala \rightarrow Pro}$ is the α chain variant of hemoglobin, first detected in a hemolysate of a boy 23 year old living in Karachi by Ahmed et al.-(1986) [123]. The carrier has no clinical problems. The mother and his two sisters showed no abnormal hemoglobin. Electrophoretically Hb-Karachi found to be a slow migrating variant as

compare to Hb-A. The chromatography of globin in the presence of 8M urea resolved only α and β chain. The fingerprinting of tryptic peptides showed change in position of peptide βTp1.

The amino acid sequence of the native chain established by automatic Edman degradation in a liquid phase sequencer upto 30 residues and by manually till 10 residues. Sequence revealed that alanine at α5 (A3) exchanged with proline. No trace of alanine was observed at this step confirms the hemoglobin to be homozygous.

Alanine is ambivalent i.e. neither so hydrophobic that it always points internally in the hemoglobin molecule nor so hydrophillic as to point externally. Alanine in the 5th position of the α chain corresponding to the 3rd residue of A helix, is oriented towards the surface of molecule. Its substitution by proline which has the same ambivalent characteristics may not produce any change in structure function relationship. The carbon of the side chain of proline loops back to make a second connection to its amino nitrogen producing a bend in the main polypeptide chain. This is in apposition to the A2 proline residue in the normal hemoglobin. The only other mutation in the helix A3 position reported is Hb-J. Toronto [124] in which alanine is replaced by strongly hydrophillic aspartic acid. At helix A4 as many as 5 mutations are known [125-129]. This position is involved in the salt bridge with α127(H10) Lys residue. Substitution in this position

resulted in higher oxygen affinity but the Bohr effect and cooperativity were comparable to Hb-A. The position A3 is not involved in the function of the molecule as such changes with ambivalent and hydrophillic amino acid may not effect the physiological function of the hemoglobin.

## 4.3: HEMOGLOBIN ANDREW-MINNEAPOLIS

Hemoglobin Andrew-Minneapolis $\alpha_2^{144\text{Lys} \rightarrow \text{Asn}}\beta_2$ is a variant identified in American family by Zak et al.-1974 [19]. We are reporting it in a German family from Berlin.

The propositus a 47 year old man with an elevated glycosylated hemoglobin (17.1% of total), determined with a commercially available micro column set. Anamnestically important at the age of 15 year, a perforated ulcus duodeni. At that time his raised hemoglobin, elevated red cell number and high PCV were conspicuous. Another ulcus followed 12 year later. At the age of 31 he suffered from a chronically pancreatitis. "For the diagnosis of the cause of its erythremia, an examination of his bone marrow was undertaken, which resulted in the suspicion of a subtype of polycythemia vera. A few months later his polyglobia was interpreted as a symptomatic one as the result of his multiple donation of blood"*.

Isoelectric focusing of hemolysate showed two abnormal hemoglobin bands

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* Report from Dr. Wolfgang Herold, Arzt fur Laboratoriumsmedizin internist, Transfusionsmedizin im Krankenhaus Am Urban, Berlin, West Germany.

migrated more towards anode than Hb-A. On disc elcctrophoresis only one abnormal band was observed. Separation of hemoglobin components by ion-exchange chromatography on DEAE-Sephacel column showed presence of an abnormal component, confirming the result of the disc electrophoresis. The band moving more towards anode detected by isoelectric focusing may also be an artifact due to aging of sample.

The abnormal peptide was isolated by RP-HPLC, its amino acid composition revealed it to be a chymotryptic peptide of βTp14/15. The amino acid sequence determined in a gas-phase sequencer revealed that lysine at β144(HCl) is substituted with asparagine to which the normal βTp15 dipeptide tyrosil-histidine is linked. This confirmed it to be a case of Hb Andrew-Minneapolis.

The variant has high oxygen affinity; hence hemoglobin transfers less oxygen to the tissues as compared to Hb-A and results in tissue hypoxia with compensatory increase of erythropoiesis. The stimulation of erythropoietin often causes a remarkable polyglobia. Another hemoglobin variant Hb Mito [20] at the same position β144(HCl) has aspartic acid. These substitutions are more acidic as compared to normal peptide.

## 4.4: CARNIVORA HEMOGLOBINS

The family Felidae (Cat and allies) has two living subfamilies, the Felinae and Acinonychinae. The subfamilies Proailurinae, Nimravinae, Machairondotinae and Hyainailourinae presently exist only as fossils.

The subfamily Felinae has two genera. The genus Uncia with one specie snow leopard (Uncia uncia) and the genus Panthera with four species leopard (Panthera pardus), jaguar (Panthera onco), tiger (Panthera tigris) and lion (Panthera leo).

Hemoglobin from the order Carnivora in general and the family Felidae in particular have not been investigated in detail. In the present study we have characterized the globin chains from jaguar and leopard, representative of the family Felidae.

Disc electrophoresis of hemolysate shows two hemoglobin components namely Hb-I and Hb-II. Hemoglobin from both animals were found to have the identical mobility, however they differ in concentration. In jaguar the ratio of two components (Hb-I:Hb-II) was 1:1, whereas in leopard about 8:2.

Triton gel electrophoresis revealed that the hemolysate contains three globin chains. The structural differences in the two hemoglobin components are confined to the β chains.

Separation of globin chains by chromatography on CM-Cellulose in the presence of 8M urea resulted in the separation of three peaks. However, peak corresponding to βII was found to be contaminated with the α chain. All the three chains were separated in their pure state on RP-HPLC. These results are in agreement with Triton electrophoresis.

### 4.4.1: Primary structure of hemoglobin

The complete primary structure of the hemoglobins was established by sequencing the N-terminal part of globin chain up to 42 residues and by sequencing the tryptic peptides.

The sequences compared for homology with that human Hb-A revealed alteration of 22 residues in the α, 29 in the βI and 28 in the βII chain. The exchanges which are distributed over the entire length of the molecule result in the alteration of 10 functionally important positions, $\alpha_1\beta_1$, $\alpha_1\beta_2$, heme contact points, DPG binding sites and the amino acid which are responsible for the Bohr effect.

### 4.4.2: Binding sites for deoxyhemoglobin

The heme binding sites for the hemoglobin (Table.6.1) in the α and β chain are present inside the molecule and the amino acid residues are mostly hydrophobic. In jaguar and leopard β70 (E14) alanine is replaced with serine; this substitution is found frequently in the mammalian hemoglobin. The heme binding sites in the chain correspond to human hemoglobin.

Table 6.1: Heme binding sites for deoxyhemoglobin. Only the exchanges are given for jaguar (Po) and leopard (Pp) hemoglobins.

| α | Hb-human | | Po-β | Pp-β | Hb-human | | β |
|---|---|---|---|---|---|---|---|
| 42 | C7 | Tyr | | | Phe | C7 | 41 |
| 43 | CD1 | Phe | | | Phe | CD1 | 42 |
| 45 | CD3 | His | | | His | E7 | 63 |
| 46 | CD4 | Phe | | | Lys | E10 | 66 |
| 58 | E7 | His | | | Val | E11 | 67 |
| 61 | E10 | Lys | **Ser** | **Ser** | Ala | E14 | 70 |
| 62 | E11 | Val | | | Phe | E15 | 71 |
| 66 | E15 | Leu | | | Phe | F1 | 85 |
| 80 | F1 | Leu | | | Leu | F4 | 88 |
| 83 | F4 | Leu | | | Leu | F7 | 91 |
| 86 | F7 | Leu | | | His | F8 | 92 |
| 87 | F8 | His | | | Leu | FG3 | 96 |
| 91 | FG3 | Leu | | | Val | FG5 | 98 |
| 93 | FG5 | Val | | | Asn | G4 | 102 |
| 97 | G4 | Asn | | | Phe | G5 | 103 |
| 98 | G5 | Phe | | | Leu | G8 | 106 |
| 101 | G8 | Leu | | | Val | H15 | 137 |
| 132 | H15 | Val | | | Leu | H19 | 141 |
| 139 | H19 | Leu | | | | | |

The $\alpha_1 \beta_2$ binding sites (Table 6.2) which are responsible for the construction of tetrameric structure are found to be invariant in many species. Substitution at these positions alter the heme-heme interaction, results in the impairment of the respiratory function. In jaguar and leopard one substitution is located at β43 (CD2) Glu/Gln. The binding sites in the α chain is identical with human hemoglobin.

For the dimeric structure α1β1 contact (Table 6.3) is very important. Four exchanges have been located in jaguar and leopard hemoglobins at α34 (B15) Leu/Cys, α111 (G18) Ala/Cys and in the β chain β123 (H1) Thr/Asn and β125 (H3) Pro/Gln.

Bunn-1971 [130] has divided mammalian hemoglobins into two group on the basis of substitution at these binding sites.

**(a)** The hemoglobin with low intrinsic oxygen affinity show no effect from this organic phosphate. In this group are the hemoglobin in which β1(NA1) is deleted and β2(NA2) is replaced with methionine e.g bovine, sheep and goat [131-133].

**(b)** The hemoglobin with high intrinsic oxygen affinity modulated with 2,3-diphosphoglycerate. Representative of this group are the hemoglobin from human and most of the other mammalian.

Table 6.2: $\alpha_1\beta_2$ binding sites for human deoxyhemoglobin. The only exchanges are given for jaguar (Po) and leopard (Pp) hemoglobins.

| α | Hb-human | | Po-β | Pp-β | Hb-human | | β |
|---|---|---|---|---|---|---|---|
| 37 | C2 | Pro | | | His | HC3 | 146 |
| 38 | C3 | Thr | | | Tyr | HC2 | 145 |
| 40 | C5 | Lys | | | Leu | G7 | 105 |
| 41 | C6 | Thr | | | Asn | G4 | 102 |
| 42 | C7 | Tyr | | | Glu | G3 | 101 |
| 44 | CD2 | Pro | | | Asp | G1 | 99 |
| 88 | F9 | Ala | | | Val | FG5 | 98 |
| 91 | FG3 | Leu | | | His | FG4 | 97 |
| 92 | FG4 | Arg | **Gln** | **Gln** | Glu | CD2 | 43 |
| 94 | G1 | Asp | | | Phe | C7 | 41 |
| 95 | G2 | Pro | | | Arg | C6 | 40 |
| 96 | G3 | Val | | | Trp | C3 | 37 |
| 97 | G4 | Asn | | | Pro | C2 | 36 |
| 140 | HC2 | Tyr | | | Tyr | C1 | 35 |
| 141 | HC3 | Arg | | | VAl | B16 | 34 |

Table 6.3: α1β2 binding sites for human deoxyhemoglobin. Only exchanges are given for jaguar (Po) and leopard (Pp) hemoglobins.

| α | Hb-human | | Po-α | Pp-α | Po-β | Pp-β | Hb-human | | β |
|---|---|---|---|---|---|---|---|---|---|
| 31 | B12 | Arg | | | | | Gln | H9 | 131 |
| 34 | B15 | Leu | **Cys** | **Cys** | | | Ala | H6 | 128 |
| 35 | B16 | Ser | | | | | Gln | H5 | 127 |
| 36 | C1 | Phe | | | **Gln** | **Gln** | Pro | H3 | 125 |
| 103 | G10 | His | | | | | Pro | H2 | 124 |
| 106 | G13 | Leu | | | **Asn** | **Asn** | Thr | H1 | 123 |
| 107 | G14 | Val | | | | | Phe | GH5 | 122 |
| 110 | G17 | Ala | | | | | Gly | GH2 | 119 |
| 111 | G18 | Ala | **Cys** | **Cys** | | | His | G18 | 116 |
| 114 | GH2 | Pro | | | | | Ala | G17 | 115 |
| 117 | GH5 | Phe | | | | | Cys | G14 | 112 |
| 118 | H1 | Thr | | | | | Asn | G10 | 108 |
| 119 | H2 | Pro | | | | | Met | D6 | 55 |
| 122 | H5 | His | | | | | Tyr | C1 | 35 |
| 123 | H6 | Ala | | | | | Val | B16 | 34 |
| 126 | H9 | Asp | | | | | Arg | B12 | 30 |

Our results show that the hemoglobins from jaguar and leopard have two alteration at these key positions. The βI which has blocked N-terminus and Phe in place of His at β2 (NA2), while the βII chain has free Gly as a N-terminal residue and substitution at β2 (NA2) is identical with that of βI, both of these sites play a key role in terms of determining of ease of loading and unloading of oxygen. The substitution of hydrophyllic residue β(NA2) His with hydrophobic Phe should result in the alteration of the secondary structure as Phe would tend to take an interior position in the molecule Perutz and Imai-1980 [134]. The Table 6.4 summarizes the alteration observed at these four binding sites in different mammalian hemoglobins. Beside 2,3-DPG the other hetrotrophic ligands are proton Bohr effect, chloride and carbondioxide which decreases the oxygen affinity of the hemoglobin molecule.

Table 6.4: Mammalian hemoglobins with alteration at contact points for 2,3-diphosphoglycerate.

| Mammal | β1(NA1) | β2(NA2) | β82(EF6) | β143(H21) |
|---|---|---|---|---|
| Human | Val | His | Lys | His |
| Mountain Zebra | Val | Gln | Lys | His |
| Low land tapir | Val | Glu | Lys | His |
| Armadillo | Val | Asn | Lys | His |
| Ring tailed lemur | Thr | Phe | Lys | His |
| Brown lemur | Thr | Leu | Lys | His |
| Cat (βA) | Gly | Phe | Lys | His |
| Cat (βB) | Ac-Ser | Phe | Lys | His |
| Jaguar (βI) | Ac-Ser | Phe | Lys | His |
| Jaguar (βII) | Gly | Phe | Lys | His |
| Leopard (βI) | Ac-Ser | Phe | Lys | His |
| Leopard (βII) | Gly | Phe | Lys | His |
| Bovine | - | Met | Lys | His |
| Grand galogo (βI) | Val | His | Cys | His |
| European hedgehog | Val | His | Lys | Ala |

### 4.4.3 Blocked N-terminal chain

The occurrance of two types of β chain one with blocked acetylated N-terminal and other with free N-terminus appear to be a common feature in one or major hemoglobin that are found in the blood of the family Felidae, Taketa et al.-1971 [135]. It is now found in the large members of the genera Felis, Acinoxy and Panthera Taketa-1974 [60]. In vitro experiments are suggestive of the presence of an acetyl transferase in the intermediate stages or translation as a possible clue to N-terminal acetylation Kaston-Jolly et al.-1979 [136]. It shows that the enzyme has a high degree of specificity as only N-terminal serine residue has been acetylated whereas free glycine is found as the N-terminal of βII globin chain. An interesting result of our study is that the major beta chain (βI) is found to be blocked due to the N-terminal acetylation of serine while in the cat, a member of the same family, the minor beta (βB) chain has acetylated N-terminus serine.

### 4.4.4: Evolutionary relationship of globin gene

About 425-500 million years before the original gene for globin was duplicated and 300-500 million years ago separated in to α and β chain of different chromosomes Goodman et al.-1976 [137], Goodman-1982 [138], the mammalian was separated from reptile and birds from that time. The duplication of α gene took place about 300 million years ago. About 90 million years existed the original form of the mammalian globin gene, from which bring upto date exist gene cluster.

The evolution occurred according to Zuckerkandal and Pauling [78], divergent at constant rate. Goodman gave a period of 300 million years for a constant rate on "molecular clock". According to Goodman-1975 [85] mutation of one amino acid required about 1.5 million years. Kimura-1982 [139] gave a period of one million years and Wilson-1977 [140] gave a different evolution rate for the α chain (1.2 million years) and for the β chain (1.1 million years). The differences considered the data that in mammalian mostly more substitution are found in the β chain.

Comparison of the amino acid sequence with that of other available sequences of the family Felidae revealed high degree of homology both in α chain and β chain. The minimum number of amino acid differences found after comparing the available sequences from the family Felidae are presented in Figure-5.6. By taking the mutation rate 1.5 million years per amino acid residue according to Goodman-1982 the values arises for the human and different Felidaes are given in Figure-5.7.

### 4.4.5: Identical amino acid sequence

The result of our studies on the primary structure of hemoglobin from jaguar and North Persian leopard revealed that the all three globin chains are identical in their amino acid sequence.

Fig-5.6: The minimum amino acid exchanges in α, βI and βII globin chains of the human and representatives of the family Felidae. (A) α-globin chain (B) βI/βII globin chain.

**(A)**

| | Human | Jaguar | Leopard | Lion | Tiger | Cat |
|---|---|---|---|---|---|---|
| Human | 0 | | | | | |
| Jaguar | 22 | 0 | | | | |
| Leopard | 22 | 0 | 0 | | | |
| Lion | 23 | 1 | 1 | 0 | | |
| Tiger | 23 | 2 | 2 | 3 | 0 | |
| Cat | 22 | 9 | 9 | 10 | 11 | 0 |

**(B)**

| | Human | Jaguar | Leopard | Lion | Tiger | Cat |
|---|---|---|---|---|---|---|
| Human | 0 | | | | | |
| Jaguar | 29/28 | 0 | | | | |
| Leopard | 29/28 | 0 | 0 | | | |
| Lion | 29/28 | 0 | 0 | 0 | | |
| Tiger | 29/28 | 0 | 0 | 0 | 0 | |
| Cat | 25/28 | 6/4 | 6/4 | 6/4 | 6/4 | 0 |

Fig-5.7: Estimated values for the separation of human and members of the family Felidae in million years.

| | Human | Jaguar | Leopard | Lion | Tiger | Cat |
|---|---|---|---|---|---|---|
| Human | 0 | | | | | |
| Jaguar | 118.5 | 0 | | | | |
| Leopard | 118.5 | 0 | 0 | | | |
| Lion | 120.0 | 1.5 | 0 | 0 | | |
| Tiger | 120.0 | 3.0 | 3.0 | 4.5 | 0 | |
| Cat | 112.5 | 28.5 | 28.5 | 30 | 31.5 | 0 |

Among the order Carnivora the only other known example of identical sequence reported is the family Ursidae where the hemoglobin from Asiatic black bear, Polor bear and Malyan sun bear showed Identical primary structure [141]. Table 6.5 shows a list of known hemoglobins having identical amino acid sequence.

Table 6.5: Identical hemoglobin chains found in different species.

| Species | Identical chains |
|---|---|
| Homo sapiens, Pan paniscus, Pan troglodytes [142] | α,β |
| Homo sapiens, Pan troglodytes [142,143] | γ |
| Cebus capucinus [144], Cebus apella [145] | β |
| Ateles geoffroyi, Ateles fusciceps [146] | δ |
| Ateles geoffroyi, Ateles belzebuth, Ateles paniscus [147] | β |
| Saguinus oedipus [148], Saguinus nigricollis [146] | β |
| Macaca sepeciosa cuvier [149], Macaca fuscata fuscata [150] | β |
| M.nemestrina [151], M.fascicularis [152] | |
| Macaca fuscata fuscata [153], Macaca mulatta [154] | α |
| Macaca fuscata fuscata [155], Macaca mulatta [156] | γ |
| Camelus dromedarius, Camelus ferus bactrianus [157] | α,β |
| Lama glama, Lama vicugna, Lama pacos [158] | β |
| Bos primigenius, Bos grunniens [159] | γ |
| Bos taurus (A-Allele) [131,132], Bos grunniens [160] | βI |
| Equus hemionus kulan, Equus zebra [161] | β |
| Ursus tibetanus, Ursus maritimus, Helarctos malayanus [141] | α,β |
| Panthera leo [68], Panthera tigris [162] | β |
| Panthera onco [67], Panthera pardus sexicolor [163] | α,β |
| Branta canadensis, Cygnus olor [164] | β |
| Gyps rueppellii, Aegypius monachus [165], Trigonoceps occipitalis | β |
| Aegypius monachus [165], Trigonoceps occipitalis | $\alpha^D$ |

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