HAEMOGLOBIN SAINT LOUIS β28 (B10) LEUCINE → GLUTAMINE
A new unstable haemoglobin only present in a ferri form

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1. Introduction

Up to the present, more than twenty amino acid substitutions in the haem pocket have been described [1] leading to haemoglobins M (slightly unstable), or to unstable haemoglobins (often partially oxidized).

In this paper an unstable and oxidized haemoglobin, haemoglobin Saint Louis, with a substitution localized close to the β haem group is described. Since its properties are quite different from those of abnormal haemoglobins previously described, this haemoglobin seems to be unique.

2. Material and methods

The abnormal haemoglobin was detected in hospital Saint Louis at Paris in a patient twenty years-old, born in the north of France, bearing a chronic haemolytic anaemia associated with cyanosis. Parents and siblings were found to have a normal haemoglobin; since no indication of false paternity was found, a mutation event is likely.

Blood was collected on heparin; routine techniques were used for the preparation and electrophoresis of haemolysates (on starch gel and on cellulose acetate strips), and for the detection of an unstable component [2–4]. Spectra were measured on a Unicam SP 800 spectrophotometer.

The abnormal haemoglobin was isolated by starch block electrophoresis. The abnormal β chain was precipitated by PMB treatment [3], and purified by column chromatography on CMC in 8 M urea [4, 5]. Fingerprints of tryptic hydrolysates of S-aminoethylated abnormal β chains were performed on cellulose Whatmann CC41 thin-layers [6].

Peptides were isolated and purified by ion-exchange resin column chromatography as previously described [4, 7]. Amino acid compositions were determined with a Bio Cal BC 200 amino acid analyzer.

Automatic Edman degradation was carried out in a Beckman 890 B sequencer, according to Edman and Begg [8] with the quadrol double cleavage program D. The thiazolinones were converted to PTH amino acids, and these were identified by thin-layer chromatography on miniature silica gel sheets as described in [9].

Abbreviations:
PMB: *p*-chloromercuribenzoate;
CMC: carboxymethyl-cellulose;
PTH: phenylthiohydantoin;
EPR: electron paramagnetic resonance.

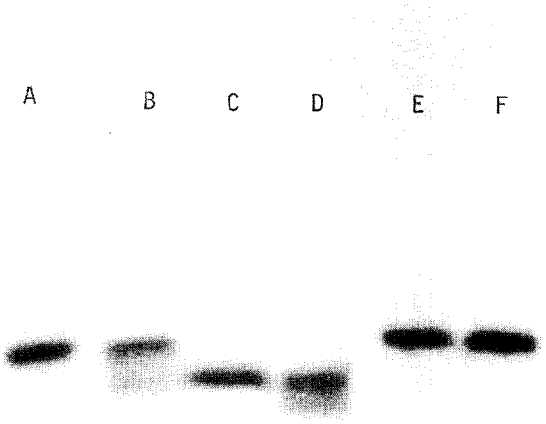

Fig. 1. Starch gel electrophoresis of haemoglobin Saint Louis and of normal haemoglobin as reference after application of 7 V/cm for 2.5 hr. Electrophoresis was performed in a EDTA–boric acid–Tris discontinuous buffer [2].
Samples are:
a) Normal adult haemolysate;
b) patient's haemolysate;
c) normal after addition of ferricyanide;
d) patient's haemolysate after addition of ferricyanide;
e) normal haemolysate after addition of KCN;
f) patient's haemolysate after addition of KCN.

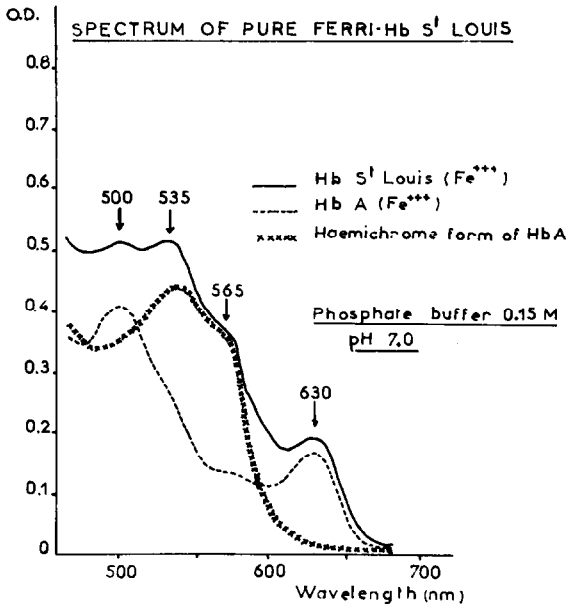

Fig. 2. Comparison of spectra between 500 and 700 nm of purified haemoglobin Saint Louis after treatment with potassium ferricyanide at pH 7.0 (——), normal haemoglobin (----) and normal haemichrome obtained by treatment with imidazole [11]. The spectrum of oxidized haemoglobin Saint Louis exhibits four maxima at 630, 565, 536 and 500 nm; this suggests that the abnormal haemoglobin is present simultaneously in ferri and haemichrome forms. This finding is consistent with EPR data [12].

3. Results and discussion

3.1. *Electrophoretic properties of haemoglobin Saint Louis*

At pH 8.6 the electrophoretic pattern of fresh patient's haemolysate (fig. 1) was analogous to that of a mixture of 70% of normal and 30% of ferri-haemoglobins. After addition of KCN a normal pattern was observed. After oxidation by ferricyanide, in addition to normal methaemoglobin a slower band representing 30% of the total haemoglobin was found. The extra positive charge of this band can be suppressed by decreasing the pH to 7.4. Thus the electrophoretic patterns of haemoglobin Saint Louis are different from those of normal methaemoglobin, and of the haemoglobins M [10].

The spectrum of this band (fig. 2) was similar to that described by Rachmilewitz [11] for haemichromes (low spin compounds in which the sixth coordinance of the haem iron is bound to an imidazole group). Preliminary studies published elsewhere [12] indicate that the extra positive charge unexplained by the amino acid substitution (see below) is most probably due to haemichromes which were found in EPR studies.

Table 1
Amino acid composition of the unstable β chain precipitated by PMB [3] and purified by CMC chromatography in urea [5].

	Expected	Found
Lysine	11	11.2
Histidine	9	8.7
Arginine	3	3.1
Aspartic acid	13	13.4
Threonine	7	6.9
Serine	5	4.8+
Glutamic acid	11	12.3
Proline	7	6.7
Glycine	13	13.0
Alanine	15	15.4
Half cystine	2	?
Valine	18	16.2°
Methionine	1	0.4
Isoleucine		
Leucine	18	16.4
Tyrosine	3	2.8
Phenylalanine	8	7.9
Trytophan	2	2•

Except when stated otherwise, results are based on the mean of duplicate analyses of 22 and 72 hr hydrolysates in 5.7 N HCl–0.009% phenol in vacuum at 110°C.

+ Extrapolation to zero time
° After 72 hr hydrolysis.
• Estimated by specific staining on fingerprints.

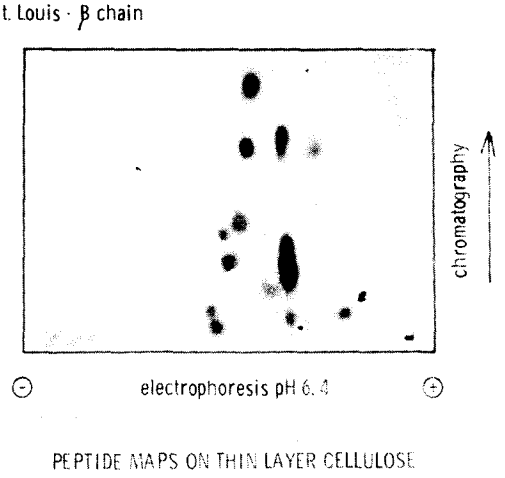

Fig. 3. Fingerprints of tryptic digests of S-aminoethylated β Saint Louis chains were made on cellulose thin-layer plates 20 × 20 cm according to Blomback [5]. A 20 V/cm electrophoresis in pyridine, acetic acid, water (100:4:100, v/v/v) was followed by ascending chromatography in n-butanol, acetic acid, water, and pyridine (40:8:21:32, v/v/v/v). Peptides were stained by ninhydrine. In the mutated β chain all the peptides were found in the normal place except for the βT3 peptide which has a weakly modified R_f value (indicated by an arrow).

The abnormal haemoglobin was found to be thermo-unstable; reaction with PMB gave rise to the specific precipitation of the mutated β chain.

3.2. *Determination of the structure of the abnormal β chain*

The abnormal β chain precipitated by PMB had a behaviour identical to that of a normal β^A chain on CMC column chromatography in urea, indicating that its charge was normal. Its amino acid composition showed that it contained one leucine residue less, and one glutamic residue more than of a normal β chain (table 1).

Fingerprints of the tryptic digest of aminoethylated β Saint Louis chains showed that all the peptides were in a normal position except for the βT3 peptide; this peptide had a normal electrophoretic mobility, but a lower R_f value indicating a higher polarity (fig. 3).

The elution pattern of the tryptic peptides isolated by ion exchange resin column chromatography [7] was normal. All the peptides have a normal amino acid composition, except for peptide βT3, in which the substitution Leu → Glx occurred in position 28, since there is only one leucine residue in this position in this peptide (table 2).

3.3. *Nature of the substitution Leu → Glx*

Indirect evidence for the Leu → Gln instead of the Leu → Glu substitution is given by the normal chromatographic and electrophoretic behaviour of the whole β Saint Louis chain and of the peptide containing the amino acid substitution. In addition the genetic code would only allow the replacement of leucine by glutamine by a single base substitution.

Residue β28 occupies the eleventh position in the peptide βT3; it cannot be reached easily by manual Edman degradation, exopeptidases, or specific chemical and enzymatic cleavages. Direct evidence for the nature of the Glx β28 was given by automatic Edman degradation [8] of 36 residues of intact β Saint Louis chain: a glutamine was found in the 28th position.

Table 2
Amino acid composition of the mutated peptide.

Amino acid	Normal βT3	βT3 found in Hb Saint Louis
Arg	1	0.8
Asp	2	2.0
Glu	2	3.1
Gly	3	2.6
Ala	1	0.9
Val	3	2.6
Leu	1	0.1

The abnormal β chain was purified, aminoethylated and then digested by trypsin. The elution pattern of the peptides isolated by column chromatography on Aminex A5 [12] was normal. Each peptide was further purified by column chromatography on another ion-exchanger (AG 50 W X 2, AG 50 WX 4 or AG 1 X 2 [4]; after hydrolysis at 110°C, for 22 hr, in evacuated sealed tubes in 5.7 N HCl–0.009% phenol, the amino acid compositions were determinated on a Bio Cal BC 200 amino acid analyzer. Residues representing less than 0.1 were omitted. The amino acid composition of all the peptides was found to be normal, except for the peptide βT3. In this abnormal peptide one leucine residue was missing and an additional glutamic acid or glutamine residue was present. Since in the normal peptide there is only one leucine residue (number 28), the substitution of a leucine for a glutamic acid or a glutamine occurred in this position in the β Saint Louis chain.

3.4. *Mechanism of instability*

Two abnormal unstable haemoglobins mutated in β28 (B10) position have been described. In haemoglobin Genova β28 Leu → Pro [4], the disruption of the B helix by the new proline residue is the mechanism involved in the instability. Such a mechanism is not involved for haemoglobin Saint Louis since glutamine can be incorporated into an α helix without any disruption.

The B10 residue has been shown to be in an internal position, with its side chain pointing towards the distal histidine E7 [13]. The interpretation of Perutz is that the side chain of glutamine B10 can be linked by a hydrogen bond to the histidine E7. This would be expected to affect the tertiary structure of the haem pocket and therefore of the whole polypeptide chain leading to its instability and probably to formation of haemichrome. Such an hypothesis can be checked only by X-ray analysis. This has already been started.

4. Conclusions

In our study the methods used in the description of abnormal haemoglobins were not very efficient: the fingerprints indicate solely a weak difference at the limit of accuracy; the ion exchange chromatographic method of Jones [7] failed to give satisfactory results, since the elution pattern of peptides was normal, and since the substitution was only found after determination of the amino acid composition of all the peptides.

The nature of the substitution was elucidated by automatic Edman degradation [8]. Our results indicate that whenever necessary this method can solve the sequence of abnormal haemoglobins up to the 40th residue.

Perutz and Lehmann indicated in 1968 [14] that residue B10 was in a general position. Nevertheless, further studies on haemoglobin Genova [4, 15] gave some indirect information for the functional involvement of this residue; furthermore our results on haemoglobin Saint Louis give direct evidence for the importance of the B10 residue, closely related to F8 His which is the oxygen binding locus.

Acknowledgements

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