STRUCTURAL STUDIES OF HEMOGLOBIN SAINT ETIENNE β 92 (F8) HIS → GLN: A NEW ABNORMAL HEMOGLOBIN WITH LOSS OF β PROXIMAL HISTIDINE AND ABSENCE OF HEME ON THE β CHAINS

Y. BEUZARD, J.CL. COURVALIN, M.COHEN SOLAL, M.C. GAREL and J. ROSA
Unité INSERM de Recherches sur les Anémies, U 91, Hôpital Henri Mondor, 94 Creteil, France

and

C.P. BRIZARD and A. GIBAUD
Centre Hospitalier Régional de Saint Etienne, St. Etienne, France

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1. Introduction

In all hemoglobins and myoglobins whose primary structure has been determined, even those of carp and chironomus [1] the position F8 is invariably occupied by the amino acid histidine, which is linked covalently to the sixth coordinance of the heme iron.

Indeed among more than 130 abnormal hemoglobins studied before the description of hemoglobin Saint Etienne [2], the only example of a substitution at F8 was the case of Hyde-Park hemoglobin [3], in which the F8 histidine is replaced by tyrosine, which is strongly linked to the 6th iron coordinance, and where the iron is maintained in ferric form.

This report describes the study of the primary structure of hemoglobin Saint Etienne. The replacement of β 92 (F8) histidine by the neutral residue glutamine is accompanied by absence of heme on the β chains. This affords the first direct proof of the prediction of the Perutz model, that a covalent link is absolutely required in the binding of heme to globin.

2. Material and methods

The abnormal hemoglobin was detected in a young Caucasian boy in the town of Saint Etienne, whose slight clinical disorders have been described elsewhere [4].

The abnormal hemoglobin was isolated by DEAE Sephadex chromatography [5]. The abnormal β chain was directly obtained from total hemolysate by PHMB* precipitation according to the procedure of Rosemeyer [6] and pure abnormal β chain obtained by chromatography on CMC* in urea according to Clegg [7]. Fingerprints of tryptic hydrolysate of S-aminoethylated β chains were performed on cellulose thin layers according to Blomback [8]. Isolation and purification of the β T10 mutated peptide was performed on ion exchange resin chromatography as previously described [9]. Amino acid analyses were performed with an automatic analyser Biocal 200. Electrophoretic separation of the chains were obtained on acetate cellulose strips with a 6 M urea buffer as previously described [10].

The number of heme groups was determined spectrophotometrically [11] and also by measuring the iron content [12] of a portion of hemoglobin Saint Etienne, whose protein content was independently determined by amino acid analysis.

Abbreviations:
PHMB: *p*-hydroxymercuribenzoate; CMC: carboxymethylcellulose.

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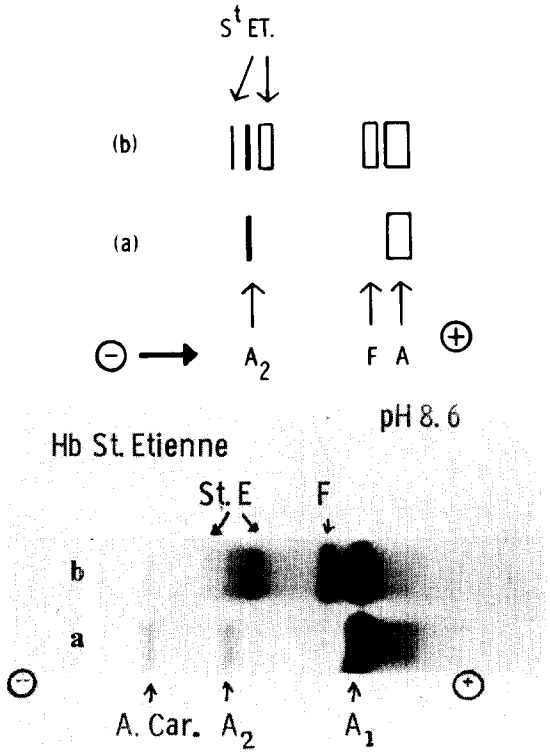

Fig. 1. Cellulose acetate strips electrophoresis of hemoglobins stained with amido-Black 10 B after application of 7 V/cm for 1.5 hr. Electrophoresis was performed in a EDTA – boric acid-Tris discontinuous buffer: 0.025 M boric acid, 0.05 M Tris, 0.7 mM EDTA, pH 8.6, for the bands and 0.031 M boric acid pH 8.6 for the tanks [13]. Samples (120 μg) are: a) normal adult hemolysate; b) Saint Etienne whole hemolysate. The proportion of the different bands was determined by densitometry of the cellulose acetate strips (Densitometer Cellomatic Sebia). Free α chain was visible on the electrophoresis after specific coloration with *o*-tolidine.

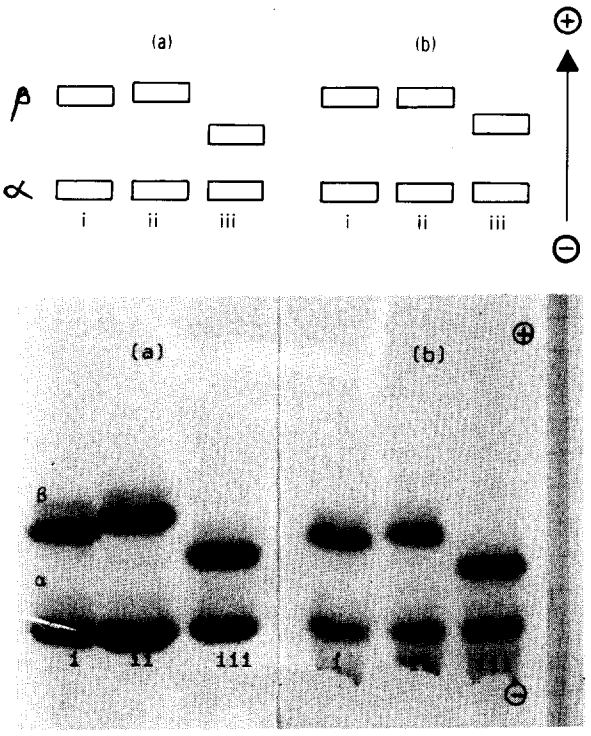

Fig. 2. Cellulose acetate strips electrophoresis of globin stained with amido-Black 10 B after application of 7 V/cm for 4 hr. Electrophoresis was performed in a discontinuous buffer [10] 6 M urea; 0.065 M Tris; 0.018 M EDTA; 0.017 M Borate; for the tanks, 6 M urea; 0.1 M Barbital for the bands. Samples were equilibrated for 1 hr before electrophoresis with the bands buffer made 0.028 M in 2-mercaptoethanol. (i) globin A; (ii) globin St. Etienne; (iii) globin S; in (a) electrophoresis at pH 7.0, in (b) electrophoresis at pH 8.0. One can see that at pH 7.0, β St. Etienne has much less than one charge difference with β^A and that at pH 8.0 it has nearly the charge of β^A. Influence of one charge difference is given by comparison between migration of β^A and β^S in these conditions.

3. Results and discussion

3.1. *Electrophoretic properties*

Electrophoresis of the hemolysate (fig. 1) shows in addition to normal hemoglobin A_1 and hemoglobin A_2, 20% of fetal hemoglobin and 25% of abnormal components migrating close to hemoglobin A_2. The abnormal hemoglobin is thermo-unstable and precipitates in presence of PHMB. A preliminary amino acid analysis of the precipitate has shown that the unstable chain was the β chain.

Electrophoresis of this β chain in a urea buffer had shown (fig. 2) that its migration at pH 8.0 was similar to the β^A chain migration, and very different from the migration of the β^S chain.

3.2. *Localisation of the substitution*

The amino acid analysis of the β Saint Etienne had shown (table 1) one histidine less, and one glutamic more than normal β^A. The fingerprints of the tryptic hydrolysate of the S-aminoethylated abnormal β chain at pH 6.5 (fig. 3) demonstrated that the β T10

Table 1
Amino acid composition of Hb β chains.

Amino acid	Hemoglobin Saint Etienne: Observed	Nearest integer	Calculated from sum tryptic peptides	Hemoglobin A - β: Nearest integer
Lysine	10.72	11	11	11
Histidine	8.07	8	[8]	[9]
Arginine	3.20	3	3	3
Aspartic acid	13.40	13	13	13
Threonine +	6.70	7	7	7
Serine +	4.60	5	5	5
Glutamic acid	12.32	12	[12]	[11]
Proline	6.80	7	7	7
Glycine	13.34	13	13	13
Alanine	15.07	15	15	15
Valine	17.83	18	18	18
Methionine	0.81	1	1	1
Isoleucine	0	0	0	0
Leucine	18.18	18	18	18
Tyrosine	2.85	3	3	3
Phenylalanine	7.68	8	8	8
Amino ethyl cysteine	1.73	2	2	2
Tryptophan•	2	2	2	2
Sum			146	146

Amino acid composition of hemoglobin β chains. Except as noted, results are based on the mean of two analyses of 22 hr hydrolysis with 5.7 N HCl in vacuum at 110°. Calculations are based on the best fit to 142 residues.
+ Extrapolated to zero time. • Determined on fingerprints.

peptide was in a more acidic position than the normal β T10 and did not give the color specific for histidine. The amino acid analysis of the abnormal β T10 isolated by column chromatography confirmed that the substitution His → Glx found in the whole β chain, occurred in position 92 (table 2), since β T10 contains just one histidine in position 92.

3.3. *Nature of the residue Glx (F8)*

Evidence that hemoglobin Saint Etienne peptide β T10 contains glutamine and not glutamic came from electrophoresis at pH 8.0 in 6 M urea (fig. 2); under these conditions, β^S (6 Glu → Val) is more basic than β^A, but β Saint Etienne shows the same mobility as β^A. So it is clear, therefore, that the alteration in β Saint Etienne involves the replacement of (F8) histidine by glutamine; this accords well with the presently accepted coding assignments which permit His → Glu NH_2 one step transition, but excludes His → Glu [14].

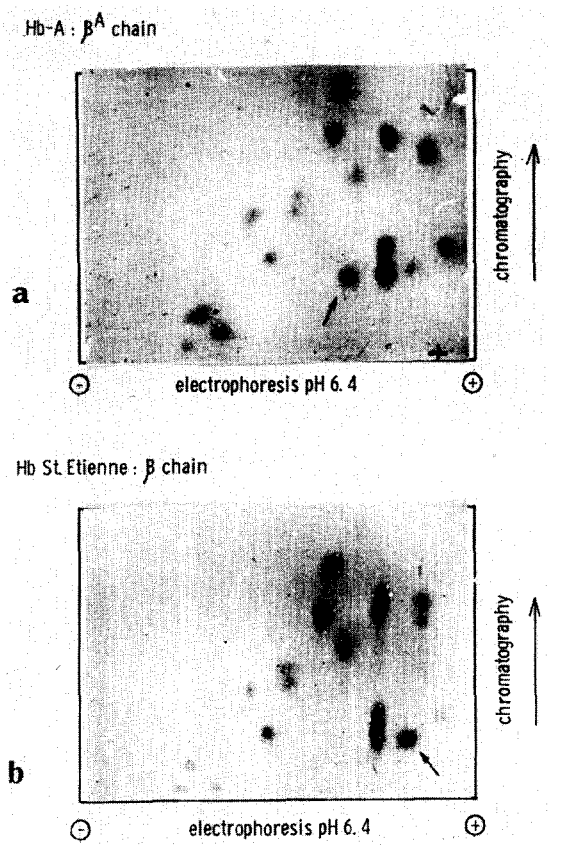

Fig. 3. Peptide maps of tryptic digests of the S-aminoethylated β chains from hemoglobin A (a) and hemoglobin Saint Etienne (b). Support medium was cellulose thin-layer plates (Whatmann CC 41) according to Blomback [8]. A 20 V/cm electrophoresis was carried out in pyridine, acetic acid, water (100/4/1000, by volume) pH 6.4 followed by ascending chromatography in n-butanol, acetic acid, water and pyridine (400/80/210/320, by volume). The peptides were stained with ninhydrine. In Saint Etienne β digests, tryptic peptide β10 is absent from its usual position and shifted to a more anodic position indicated by the arrow.

3.4. *Number of heme groups contained in hemoglobin Saint Etienne*

In hemoglobin Saint Etienne, the determination of

Table 2
Amino acid composition of the mutated peptide of hemoglobin Saint Etienne.

Amino acid	Normal β T10	β T10 found in Hb St. Etienne
LYS	1	0.97
HIS	[1]	[0.00]
ARG	–	–
ASP	1	1.06
THR +	2	1.79
SER+	1	1.10
GLU	[1]	[1.95]
PRO	–	–
GLY	1	1.05
ALA	1	1.00
VAL	–	–
AE CYS	1	0.66
MET	–	–
LEU	2	1.92
TYR	–	–
PHE	1	0.95
TRY•	–	–

Amino acid composition of the mutated peptide. Peptides β T10 were isolated on column chromatography (resin Beckman PA 35) repurified on Dowex A-G X 2 [9] and hydrolysed at 110° for 22 hr in evacuated tubes before automatic amino acid analysis. Residues forming < 0.10 of an integer are omitted.

+ Values extrapolated to zero time hydrolysis.

• Estimated from ultraviolet absorption spectra and peptide maps.

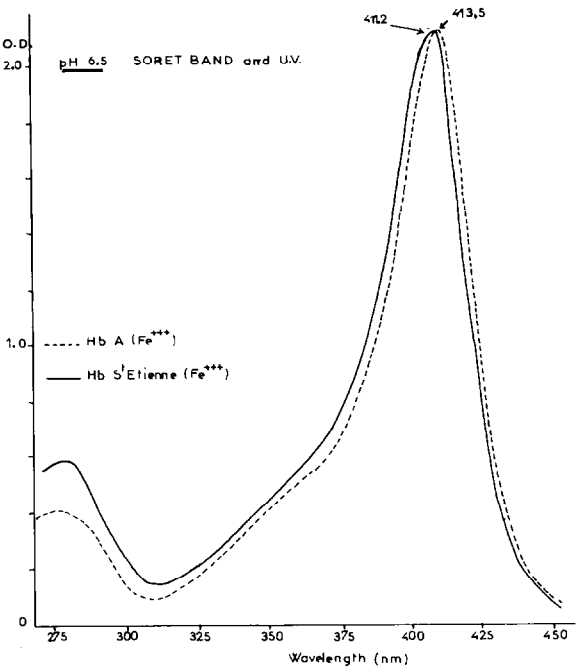

Fig. 4. Absorption spectrum of hemoglobin Saint Etienne in ferric form is shown by solid line. Dotted line represents spectrum of hemoglobin A. Absorption in the UV band of hemoglobin Saint Etienne is greater than absorption of hemoglobin A for a same absorption in the Soret band.

protein content by amino acid analysis yielded values twice those obtained when the concentration was estimated from the optical absorption of the heme groups at 540 nm, or from iron determination. The absorption spectrum of hemoglobin Saint Etienne reflected the abnormal ratio of heme to globin. A greater optical absorption in the ultraviolet region relative to the absorption at the visible wavelengths was observed in hemoglobin Saint Etienne, when compared to hemoglobin A (fig. 4). Since the loss of heme had been described during purification of some unstable hemoglobins [15] which could, thereafter, bind free heme groups added to the medium, we have tried to add heme groups to the pure hemoglobin Saint Etienne. No heme was bound (fig. 5) in these conditions, giving the proof that glutamine (F8) is absolutely unable to link heme and that hemoglobin Saint Etienne is a natural semi-hemoglobin [16, 17].

4. Conclusions

Loss of heme in abnormal hemoglobin has been explained in several ways, including the presence of a polar residue in the heme pocket allowing entrance of water, loss of heme contacts, or presence of an amino acid too large to fit in the heme pocket without distorsion of the helix [18].

Examination of the steric model of Perutz [19] shows that none of these explanations applies to the case of hemoglobin Saint Etienne. The glutamine at F8 is an apolar residue, and is therefore unlikely to interact with any nearby amino acid, and modify the structure of the F helix; more likely, the apolarity of the glutamine prevents the formation of the covalent link with heme which usually occurs at position F8.

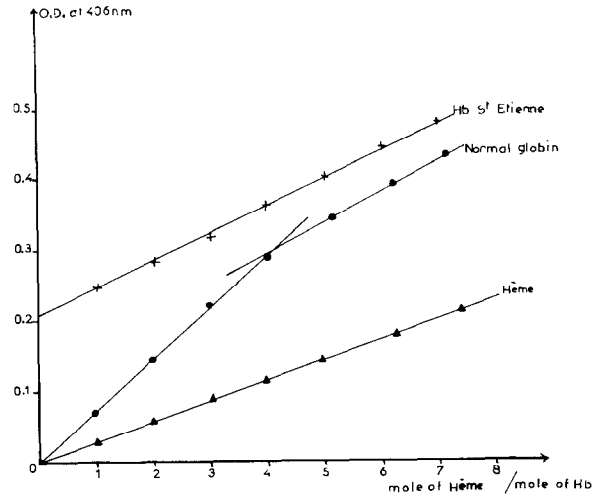

Fig. 5. Heme titration of hemoglobin Saint Etienne [15]. The absorbance was followed at 406 nm, since the absorption coefficients of ferriheme and methemoglobin are not the same at this wavelength. The change in the slope of the curve of hemoglobin A appears at full heme saturation. No change ap pears with hemoglobin Saint Etienne which does not link heme.

This is the first direct evidence that a covalent link is absolutely required in order to retain the heme molecule in hemoglobin, and that ionic hydrogen, and Van der Waals bonds are not sufficient in themselves. Still caution is required, and a definite conclusion would require X-ray analysis. It should be noted that X-ray studies, in fact, failed to confirm the first hypothesis concerning the mechanism of methemoglobinisation of hemoglobin Hyde-Park [20]. Functional and biosynthesis studies are reported elsewhere [4].

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