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The $^{M}\gamma$ chain of human fetal hemoglobin is an $^{A}\gamma$ chain with an *in vitro* modification of γ141 leucine to hydroxyleucine

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ABSTRACT

We have reanalyzed the structure of the γT-15 peptide from the minor $^{M}\gamma$ chain of human hemoglobin (Hb) F. Amino acid analysis confirmed that the Leu 141 residue was missing from position 9 of this peptide, and liquid secondary ion mass spectrometry indicated that it was replaced, not by methionine (residue mass 131) as previously believed, but by an amino acid of mass 129. By analogy with the recently reported oxidation of the corresponding leucine at position γ141 of the unstable Hb Atlanta, it appears that the $^{M}\gamma$ chain also results from the oxidation of γ141 to hydroxyleucine (residue mass 129). The finding that the proportion of the $^{M}\gamma$ chain increased when red cell lysates were prepared with carbon tetrachloride prompted us to reinvestigate the oxidation mechanism involved in the formation of β141 hydroxyleucine in Hb Atlanta. Oxidation of the β141 residue could be detected when carbon tetrachloride was used in the lysis protocol, while conversion of oxyhemoglobin to carbon monoxyhemoglobin prior to carbon tetrachloride treatment prevented oxidation. It therefore appears that the hydroxylation of Leu 141 is not an *in vivo* process in the circulating red cell. Perhaps leucine at position 141 of the β, γ, and δ chains (and at position 136 of the α chain), which forms a contact with heme and is located directly across the heme plate from the E helix, is oxidized to hydroxyleucine at a very low rate forming minute amounts of modified chains; this process is accelerated by treatment with agents such as carbon tetrachloride and prolonged exposure to air.

INTRODUCTION

In 1987, we provided evidence of the existence of a new γ chain variant of human Hb F. This $^{M}\gamma$ chain was believed to have a Leu → Met replacement at position γ141 [1]. Thus, besides the expected $^{G}\gamma$ (glycine at γ136) and $^{A}\gamma$ (alanine at γ136) chains (which are the products of duplicated γ-globin genes [2]), a third γ chain ($^{M}\gamma$) was observed. The $^{M}\gamma$ chain was detected by reversed-

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phase high-performance liquid chromatography (HPLC) [1] in red cell lysates of patients with the hereditary persistence of hemoglobin (Hb) F (HPFH), sickle cell anemia (SS), and in some cord blood samples. Its quantity was highly variable, from less than 0.2% to 38% (as percentage of total γ chains) [1]; the percentage $^{M}\gamma$ was inversely related to that of the $^{A}\gamma$ chain, consistent with the finding of alanine at position 136 of the $^{M}\gamma$ chain. The Leu → Met replacement was identified through amino acid analysis of tryptic and cyanogen bromide (CB) peptides of the isolated $^{M}\gamma$ chain; no explanation was found for its existence and "an infidelity in translation or the existence of an unrecognized γ-globin gene" was suggested [1].

More recently, a reevaluation was made of a family with three β chains in one individual, *i.e.* one normal (β^{A}) chain, one with a β75 Leu → Pro mutation which is known as Hb Atlanta (β^{At}), and one with the β75 Leu → Pro mutation and a β141 Leu → deleted which is known as Hb Coventry ($\beta^{At\text{-}Co}$) [3,4]. DNA sequence analysis failed to demonstrate the Hb Conventry deletion (β141 → 0), while mass spectrometry measurements indicated that leucine at β141 was not deleted but replaced by a novel amino acid of mass 129 Da, likely to be one of the hydroxyleucines (OH-Leu). The authors suggested that the presence of OH-Leu at β141 results from posttranslational oxidation of leucine at β141 perhaps because of the disturbance of the heme environment due to the β75 Leu → Pro replacement. The same authors report evidence for two other unstable hemoglobins with mutations on the heme side of the E helix [4], while oxidation of the leucine at β141 was also recently observed in an affected member of the original family from Atlanta with the β75 Leu → Pro mutation [5].

In reviewing both observations, it seemed possible that the substitution at position 141 of the $^{M}\gamma$ chain was erroneously identified as Leu → Met, and the possibility of posttranslational oxidation of γ141 Leu to OH-Leu needed to be evaluated.

EXPERIMENTAL

The three γT-15 peptides, labelled $^{G}\gamma$T-15, $^{A}\gamma$T-15, and $^{M}\gamma$T-15, were the same as those studied over five years ago; during this period they were stored in solution at −20°C. Red cell lysates were from blood of SS patients attending the sickle cell clinics and from one HPFH heterozygote (V.W.) who also participated in the studies conducted in 1986–1987 [1].

The amino acid compositions of the three T-15 peptides were determined with the Pico-Tag amino acid analyzer (Waters, Division of Millipore, Milford, MA, USA). Sequence analysis was made on an Applied Biosystems 471A protein sequencer with on-line gradient HPLC identification of phenylthiohydantein (PTH) amino acids. Mass spectrometry was performed using a VG70-250S double-focusing magnetic sector instrument with a liquid secondary ion source and associated cesium ion gun (VG Analytical, Manchester, UK) [6,7]. Peptides in 10% acetic acid were placed in a drop of glycerol on the probe and bombarded with a 35-keV stream of cesium ions at a current of 1–2 μA. Resulting secondary ions were accelerated from the source at 8 kV and their mass analyzed using a software-based multi-channel analysis technique. Both cesium iodine and gramicidin S were used as standards.

Red cell lysates were analyzed by isoelectrofocusing (IEF) [8] and by reversed-phase HPLC [9,10]. Lysates were prepared by adding distilled water to the washed packed red cells with and without carbon tetrachloride (CCl_4) as described before [11]. Centrifugation was at 5000 *g* for 20 min at room temperature.

Lysates of red cells from a Hb Atlanta heterozygote were made by lysing washed red cells in two volumes of water and removing the cell stroma by centrifugation at 34 000 *g* for 40 min. A 1-ml volume of lysate was then isopropanol-precipitated to isolate the unstable component which was converted to globin and digested with trypsin. In other variations of this procedure, the high-speed lysate was shaken with CCl_4 for 3 min prior to precipitation and mapping, or, alternatively, the hemolysate was first converted to car-

bon monoxyhemoglobin before shaking with CCl_4. Reversed-phase tryptic maps were run on a Waters Nova-Pak C_{18} column with the NaH_2PO_4–acetonitrile solvent system previously described [12].

RESULTS

Table I lists the amino acid compositions of the three γT-15 peptides, and compares the newest data with those established in 1987. In general, there is excellent agreement between both sets of results. An exception is the higher value for methionine obtained for the $^{M}\gamma$T-15 peptide in 1987. Sequence analysis with the protein sequencer failed to demonstrate a methionyl residue in the ninth position of this peptide (corresponding to position 141 of the intact γ chain).

Analysis of the γT-15 peptides by liquid secondary ion mass spectrometry gave the expected protonated masses of 1178 for the $^{G}\gamma$T-15 peptide and 1192 for the $^{A}\gamma$T-15 peptide, while that for the $^{M}\gamma$T-15 peptide was 1208 (Fig. 1). This information together with the data indicating that leucine is missing from this peptide confirms that leucine is not replaced by methionine (expected mass: 1210) but by hydroxyleucine (expected mass: 1208).

The earlier publication [1] reported variable quantities of $^{M}\gamma$ in red cell lysates of patients with different disorders. The relative quantity of $^{M}\gamma$ was calculated from the computerized integration value of the $^{M}\gamma$ zone (eluting in front of $^{G}\gamma$) and the $^{G}\gamma$ and $^{A}\gamma$ zones. Its accuracy is in part dependent on the total level of the fetal hemoglobin present in the lysate. We have reanalyzed samples from ten individuals with elevated Hb F levels (nine with SS and one with HPFH trait) by reversed-phase HPLC and calculated the level of $^{M}\gamma$ as percentage of total γ (= Hb F) and as percentage of γ + β (= total Hb). This information is listed in Table II. The level of this zone varies from 0.15 to 0.9% (as percentage of γ + β) and from 0.7 to 8.6% (as percentage of γ) in the nine SS patients; it does not appear to be related to the total level of γ that is present. The percentage $^{M}\gamma$ is also low in the red cell lysate of the adult subject with the HPFH heterozygosity, namely 0.2% (as percentage of β + γ) and 0.8% (as percentage of γ). The $^{M}\gamma$ zone is increased when red cell lysates are prepared with CCl_4, while shaking in air for 24–48 h also has a considerable effect (Table II).

The increase in proportion of $^{M}\gamma$ chain in lysates prepared with CCl_4 prompted us to reinvestigate the cause of oxidation of the corresponding β141 Leu to OH-Leu in Hb Atlanta. When standard lysates are prepared using CCl_4,

TABLE I

AMINO ACID COMPOSITION OF THE THREE γT-15 PEPTIDES

Values are mol/mol of peptide. The data in 1987 were obtained with an automated Beckman Spinco 121 M amino acid analyzer, and those in 1992 with a Pico-Tag amino acid analysis system (Waters, Division of Millipore).

Amino acid	$^{G}\gamma$T-15		$^{A}\gamma$T-15		$^{M}\gamma$T-15	
	1987	1992	1987	1992	1987	1992
Serine	2.75	2.77	2.80	2.78	3.08	2.65
Glycine	1.05	1.01	0.02	0	0.04	0
Arginine	1.30	1.00	1.26	1.00	1.09	1.00
Threonine	0.97	0.96	0.98	1.01	1.03	0.94
Alanine	1.99	2.09	2.95	2.99	3.09	3.00
Valine	1.93	1.99	1.91	2.08	1.95	1.95
Methionine	0.83	0.94	0.56	0.87	1.65[a]	0.93
Leucine	1.18	0.98	1.11	1.16	0	0

[a] 1.02 residues methionine and 0.63 residues methionine SO_3.

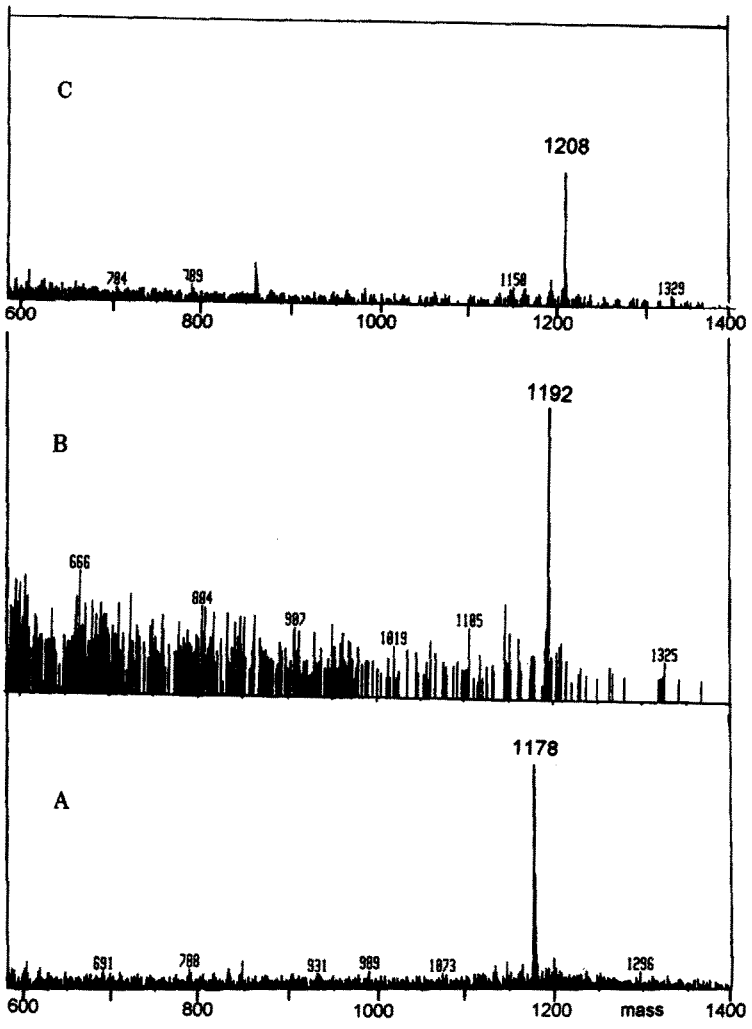

Fig. 1. Analysis of the three γT-15 peptides by liquid secondary ion mass spectrometry. (C) From the $^{M}\gamma$ chain; (B) from the $^{A}\gamma$ chain; (A) from the $^{G}\gamma$ chain.

TABLE II

LEVELS OF $^{M}\gamma$ IN THE Hb F OF ADULT SS PATIENTS OR WITH AN HPFH HETEROZYGOSITY

Samples are from SS patients; red cell lysates were prepared through lysing of isolated red cells with distilled water. V.W. is a subject with an HPFH heterozygosity who was studied before [1]; lysates were prepared in five different ways as indicated.

Subject	% γ (β + γ)	% $^{M}\gamma$ (β + γ)	% $^{M}\gamma$ (γ)	Subject	Lysate prepared with	% $^{M}\gamma$ (β + γ)	% $^{M}\gamma$ (γ)
S#G	3.9	0.2	5.1	V.W.	(1) H_2O	0.2	0.8
S#H	7.4	0.35	4.7	(26.5% γ)	(2) H_2O + CCl_4	0.9	3.4
S#D-5	5.6	0.25	4.8		Same	0.8	3.0
S#D-17	10.5	0.9	8.6		(3) H_2O + 24 h shaking	1.1	4.2
S#S-1	8.1	0.7	8.6		Same	1.0	3.8
S#S-3	5.5	0.2	3.6		(4) H_2O + CCl_4 + 24 h shaking	0.9	3.4
S#Br-5	20.6	0.15	0.7		(5) H_2O + CCl_4 + 48 h shaking	1.95	7.4
S#Br-8	30.6	0.5	1.6		Same	1.65	6.2
S#Br-15	13.2	0.65	4.9				

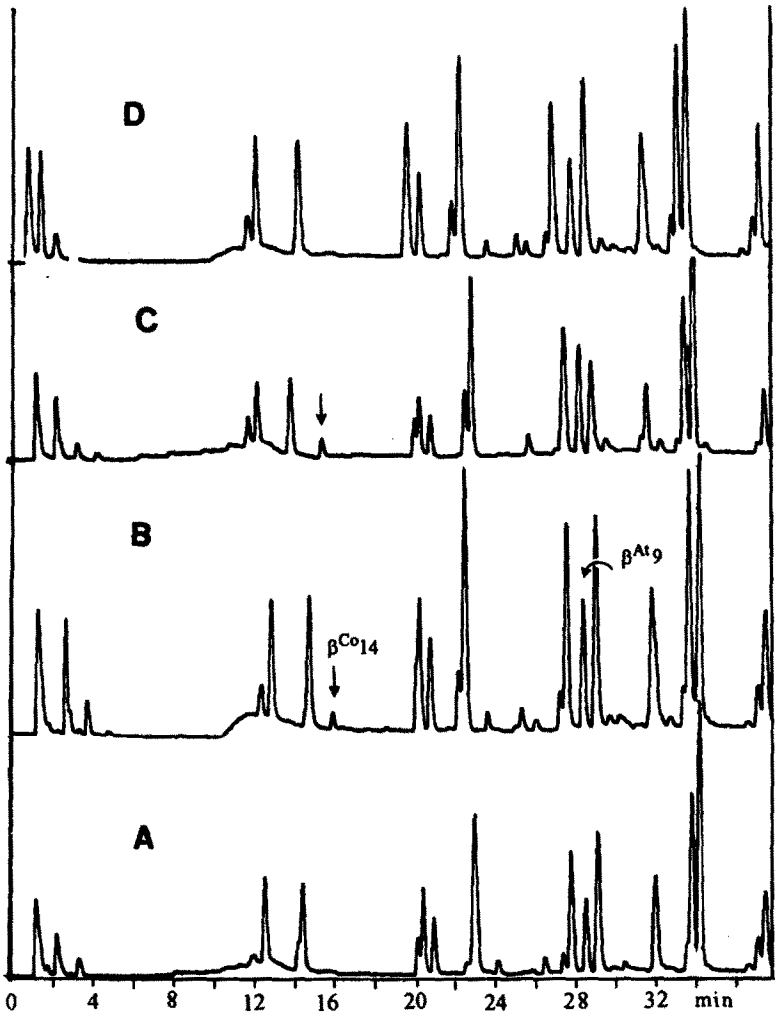

Fig. 2. Reversed-phase tryptic peptide map of isopropanol precipitates of Hb Atlanta. (A) High-speed hemolysate (note no β^{Co}T-14 peptide at 16 min); (B) high-speed lysate shaken with CCl_4 for 3 min (note appearance of β^{Co}T-14 peak); (C) standard lysate made using CCl_4; (D) high-speed lysate shaken with CCl_4 (3 min), where the hemoglobin had first been converted to carbon-monoxyhemoglobin. The abnormal peak at 28 min is β^{At}T-9 with the β75 Leu → Pro mutation.

the β^{Co}T-14 tryptic peptide containing the OH-Leu residue can be seen at 16 min in reversed-phase maps (Fig. 2C). When lysates are prepared using high-speed centrifugation instead of CCl_4, no oxidation occurs and there is no peak at 16 min (Fig. 2A). If, however, this high-speed lysate is shaken with CCl_4 prior to precipitation and mapping, then β141 again undergoes oxidation as evidenced by the return of the peak at 16 min (Fig. 2B). On the other hand, if the Hb^{At}-O is converted to Hb^{At}-CO prior to shaking with CCl_4, then oxidation is prevented (Fig. 2D).

DISCUSSION

The repeat analyses of the T-15 tryptic peptides of the three γ chains which were studied some six years ago have provided convincing evidence that the $^{M}\gamma$ chain does not carry methionine at position γ141 but an OH-Leu residue. The earlier conclusion was based on information from the amino acid composition and, to a lesser extent, the sequence analysis of the $^{M}\gamma$T-15 peptide. The recent data, however, exclude the presence of an extra methionine in this peptide, while an OH-

Leu residue was identified through mass spectrometric analysis. The inability to detect OH-Leu in the amino acid analysis is perhaps due to the formation of five- or six-membered lactone rings, dependent on the location of the hydroxyl group in the leucine residue. Thus, the $^{M}\gamma$ chain shares this unusual property with some unstable hemoglobin variants which have a similar OH-Leu residue at position 141 of the abnormal γ chain [4].

The data obtained for red cell lysates prepared with and without CCl_4 offer evidence that treatment of oxy derivatives of Hb F and of the unstable variant Hb Atlanta with this organic solvent promotes the oxidation of leucine at position 141 of the γ and β^{X} chains. It is not clear if the increase in the $^{M}\gamma$ chain zone in the chromatograms of these red cell lysates is due only to the oxidation of the leucine residue at position γ141; however, it appears to be the case since there is a molar loss of $^{A}\gamma$T-15 in the tryptic digests of the $^{M}\gamma$ chain (Fig. 3 of ref. 1). However, the $^{M}\gamma$ chain, used for the analyses in 1987 and again in 1992, was isolated from a red cell lysate which was prepared with CCl_4 and by shaking (a routine procedure at that time).

It seems reasonable to assume that oxidation of the leucine residue in position 141 of all β, δ, and γ chains, and of the leucine residue in position 136 of the α chain to give a hydroxy derivative may lead to the formation of minor components with slightly different mobilities in reversed-phase HPLC chromatograms, which are probably difficult to observe. Moreover, some chains might oxidize more easily than others. This is particularly true for the Hb Atlanta heterozygote; its β75 Leu → Pro replacement will destabilize the E helix and promote the oxidation of β141 Leu which is in contact with the heme group, and is located directly across the heme plate from the E helix [4,5]. These data indicate that oxidation occurs as an artifact of CCl_4 lysis rather than as an *in vivo* mechanism.

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