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G_γ and A_γ GLOBIN CHAINS SEPARATION AND QUANTITATION BY ISOELECTRIC FOCUSING

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SUMMARY: Isoelectric focusing in the presence of Nonidet P-40 splits human chromatographically pure γ globin chains into two bands of isoelectric points 6.95 and 6.85, respectively. The comparison of the relative proportions of the two bands with the ratios between the G_γ and A_γ non allelic chains of human fetal hemoglobin suggests that the band at pI 6.95 corresponds to G_γ and the band at pI 6.85 corresponds to the A_γ chain; the latter is the only band present in a patient with Greek type hereditary persistence of fetal hemoglobin, producing only A_γ chains. Fluorography of electrofocusing-separated radioactive γ globin chains synthesized by thalassemic reticulocytes indicates that the relative G_γ/A_γ synthetic ratios are similar to the relative amounts of G_γ and A_γ chains accumulated in the erythrocytes, suggesting that the activities for the G_γ and A_γ mRNAs decay at roughly similar rates.

INTRODUCTION: In humans two non allelic loci, closely linked to the δ and β globin genes (1), direct the synthesis of the G_γ and A_γ chains of fetal hemoglobin. The chains coded by these loci differ by having either glycine (G_γ globin) or alanine (A_γ globin) at position 136 and their ratio changes from about 3/1 in the fetus and newborn to a lower average value of approximately 2/3 in normal adults (2-6). These changes, occurring in the products of closely linked genes, provide a model for the study of cellular and molecular mechanisms in the coordinate regulation of a multigene complex.

ABBREVIATIONS: NP-40: Nonidet P-40; pI: isoelectric point; HbF: fetal hemoglobin; CMC: carboxymethylcellulose; HPFH: hereditary persistence of fetal hemoglobin; thal: thalassemia.

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The recent discovery (7) in a high proportion of normal and thalassemic individuals of a γ chain (T_γ) having threonine at position 75 instead of isoleucine, raises the possibility of the existence of a third γ locus and adds further complexity to this system. In addition, distinct G_γ/A_γ ratios may be found in a variety of inherited and acquired disorders, like hereditary persistence of fetal hemoglobin, thalassemias and other conditions, thus providing a basis for classifying these disorders into defined subtypes (1).

Unfortunately, due to the fact that the G_γ and A_γ chains differ by a neutral aminoacid substitution, quantitation of these globins can be obtained only by aminoacid analysis of the specific tryptic (T15) or cyanogen bromide (γCB3) peptide containing the variant aminoacid (2-6); this procedure, besides being time consuming and requiring large amounts of hemoglobin, makes use of a complex and expensive equipment, a fact that in practice restricts this determination to a few specialized laboratories.

In the course of studies aimed at separating human globin chains by isoelectric focusing (8) we observed that chromatographically pure γ globin chains were split into two bands upon addition of NP-40 to the gel system: we show here that these bands represent separated G_γ and A_γ chains, and that accurate quantitation of their relative proportions and synthetic rates can be readily obtained by analysis of a few µl (1-50) of blood.

METHODS: Individual human β and γ globin chains were obtained by CMC chromatography (9) of total globin prepared by the acid-acetone procedure (9) from red cell lysates from a $\delta^\circ\beta^\circ$ thalassemic homozygote and from a normal adult. The purified chains were precipitated again by acid acetone, dissolved in distilled water and lyophilized. Total globin was also prepared from red cell lysates from a patient with the Greek type of HPFH and his β thal-HPFH double heterozygous son (described in ref. 7). Lyophilized samples (50-150 µg) were dissolved in 8M urea, 3% NP-40, 10% 2-mercaptoethanol, at a concentration of 5 mg/ml, focused in 6% acrylamide gel slabs containing 8M urea, 2% pH 6-8 and 0.2% pH 3.5-10 Ampholine (LKB Produkter AB), 3% NP-40, and stained with Coomassie Brilliant Blue G-250 (8,10). The pI's were determined at room temperature (22°C) and after correction for 8M urea (11).

For protein synthesis experiments, 500 µl of heparinized blood were added to 100 µCi of lyophilized 4-5 ^{3}H leucine (114 Ci/mMole, Amersham) and incubated for 1 h at 37°C in the presence of 2 mg/ml of glucose. Globin was prepared as described (9), and γ chains purified as above. Fluorography of the stained bands was carried out as described (12,13).

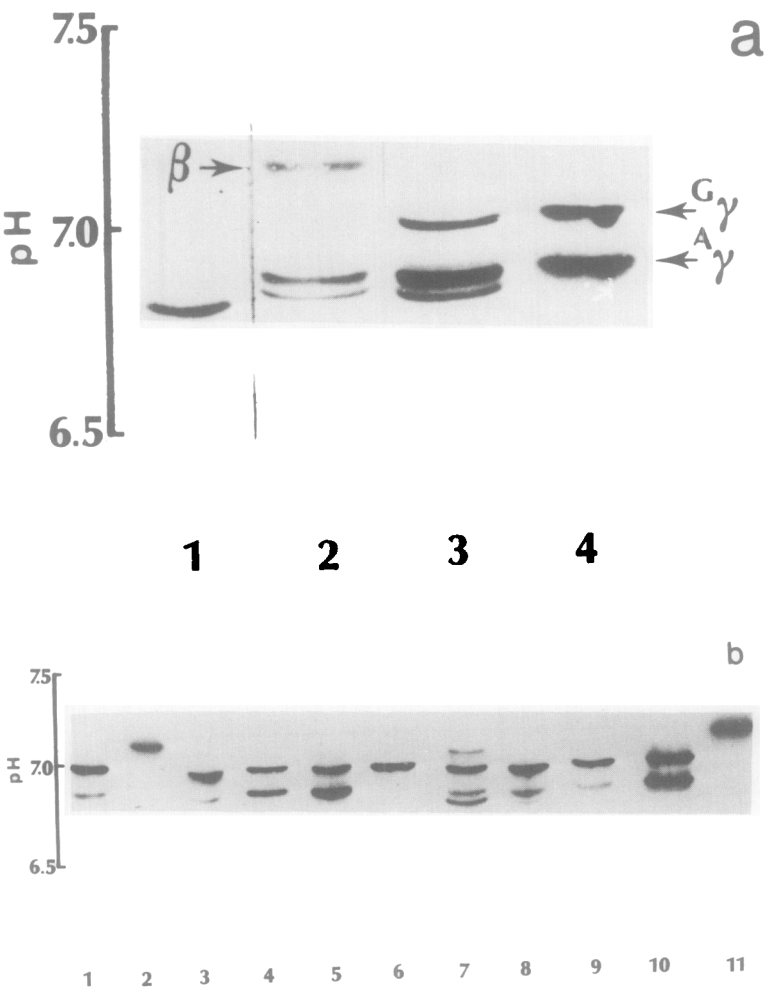
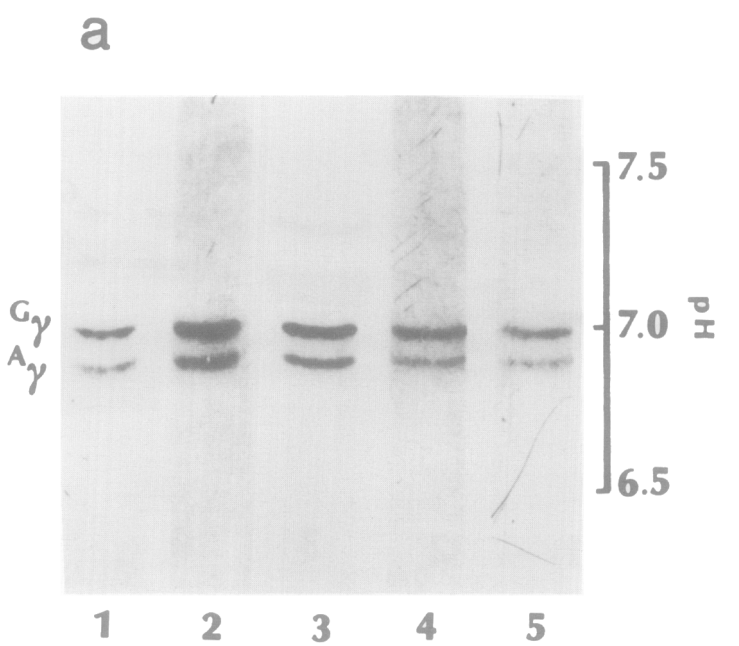


Figure 1. (a) Isoelectric focusing of human globin chains. Lane 1: γ chain from a 'fast' HbF (uncharacterized); lane 2: globins from Greek type HPFH, heterozygote; lane 3: globins from a double heterozygote for Greek type HPFH and β thalassemia; lane 4: purified γ globin chains. (b) Isoelectric focusing of purified γ globin chains (20-50 μg) from a normal fetus (lane 9), a newborn (lane 6) and from β (lanes 1,3,4,5,7,8) and δ°β° thalassemic patients (lane 10). Lanes 2 and 11: β globin chain marker.

RESULTS AND DISCUSSION: Fig. 1 (a and b) shows the isoelectric focusing separation of β and γ globin chains purified by CMC chromatography (9); while the β chains focus at a pI of 7.2, the γ chains (from a δ°β° thalassemic homozygous patient) form two well separated bands at pI's of 6.95 and 6.85.

The two γ bands might either arise from a post synthetic modification of the γ chains, or represent the individual G_γ and A_γ species; to distin-



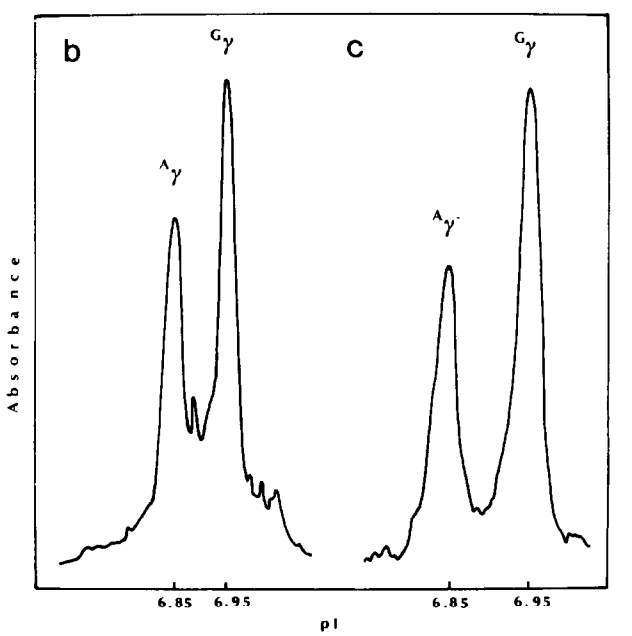


Figure 2. (a) Fluorography of CMC purified radioactive γ globin chains from thalassemic patients. Lane 1: 45 μg (900 cpm) of purified γ globin from patient 11 (see Table 1); lanes 2 and 3: 85 μg (2700 cpm) and 57 μg (1800 cpm), respectively, of purified γ globins from patient 12; lanes 4 and 5: 135 μg (1500 cpm) and 90 μg (1000 cpm), respectively, of purified γ globins from patient 13. (b) Densitometric profile of the stained gel

guish between these two possibilities, globin was prepared and analyzed as above from a patient with the Greek type of HPFH (14), having only A_γ chains, and from his β thal-HPFH double heterozygous son, having 25% G_γ and 75% A_γ (7). Whereas the double heterozygote shows two γ bands in a 1:3 ratio, the HPFH patient has only the γ band at pI 6.85 (Fig. 1 a, lane 2), which corresponds to the lower band from the β thal-HPFH (lane 3). In addition, the γ chains prepared from a newly discovered 'fast' HbF (still uncharacterized), form a single band at pI 6.80 (lane 1). These results, together with the quantitation experiments to be described below, indicate that the two γ bands obtained by isoelectric focusing are not artifacts and correspond to G_γ (upper band, pI 6.95) and A_γ (lower band, pI 6.85) chains. Amino acid analysis of the γCB3 fragments obtained from the separated bands, directly confirms the assignement of the A_γ chain to the lower band and of the G_γ chain to the upper band (in preparation).

We next explored the suitability of this technique for a reliable quantitation of G_γ/A_γ ratios: the γ globin chains were prepared by CMC chromatography of globins from normal fetal or thalassemic blood, and the purified fractions were acid-acetone precipitated and used for isoelectric focusing. Fig. 2 shows the result of this experiment and Table 1 compares the G_γ/A_γ ratios (calculated by scanning the gels and measuring the γ areas) with those obtained by the traditional method of analysis (2-7), demonstrating a good agreement between the values obtained with the two techniques. In each case the determination requires only 30 μg of γ chains, an amount contained in 0.5-5 μl of fetal or thalassemic blood. A comparably small amount (30-100 μg) of γ globin is also adequate for analysis of the radioactive product obtained by incubating thalassemic blood with ^3H leucine; fig. 2a shows the radioactive bands demonstrated by fluorography (12-13) of purified, ^3H-labelled thalassemic γ chains focused as above. By comparison of

(sample 2) obtained by scanning with a Joyce Loebl densitometer. (c) Densitometric tracing of the developed X ray film after fluorography (sample 2).

TABLE I

Comparison of the G_γ/A_γ ratios obtained by isoelectric focusing and peptide analysis.

Patient†	Condition	$\frac{G_\gamma}{G_\gamma + A_\gamma}$ by: isoelectric focusing	peptide analysis
1 (T.F.)	β° thal (intermedia)	0.77	0.74
3 (F.P.)	β^+ thal (homozygote)	0.76	0.68
4 (C.A.)	Hb Lepore (homozygote)	0.48+	-
5 (G.T.)	ß thal-HPFH (double heterozygote)	0.49	0.43
6	immature newborn (7.5 months)	0.87	0.85
7 (M.A.)	β° thal (homozygote)	0.63	0.70
8 (G.A.)	β^+ thal (homozygote)	0.80	0.82
9	fetus (3 months)	0.70	0.71
10 (T.S.)	δ° β° thal (homozygote)	0.46	0.50
11 (C.A.)	Hb Lepore (homozygote)	0.51+(0.59)=	-
12 (C.G.)	β^+ thal (intermedia)	0.61 (0.64)=	-
13 (F.G.)	β^+ thal (intermedia)	0.56 (0.62)=	-

G_γ/A_γ ratios were measured by integrating the areas under the peaks obtained by scanning either the stained gels or the X-ray autoradiographs. For determination of the ratios, the cyanogen bromide fragment (γCB3) was obtained and analyzed as described (2-7).

† Numbers 1-10 correspond to lane numbers in Fig. 1b; numbers 11-13 to lanes 1, 2-3 and 4-5 in Fig. 2a.

\+ Two different experiments.

= In parenthesis the ^3H leucine synthetic rates are given.

the scans of the stained protein zones and of the fluorographic profile (Fig. 2, b and c; Table 1, patients 11,12 and 13) it appears that the relative synthetic rates for G_γ and A_γ chains in peripheral blood reticulocytes are only slightly higher than the relative amounts of G_γ and A_γ chains accumulated in the erythrocytes, suggesting that the activities of the G_γ and A_γ mRNAs decay at roughly similar rates.

The splitting of G_γ and A_γ in presence of detergent has been called the "Nonidet effect" (8). A Gly → Ala mutation cannot possibly alter the pI of denatured γ chains, which in fact are not resolved in 8M urea gels (in the native tetramer, however, it might slightly alter the conformation leading to a slightly different pI). Therefore, we interpret the splitting and the two different pI values of the two γ chains as result of direct binding of NP-40 to them. It has in fact been reported that Triton X-100 binds to proteins (15) and NP-40 to carrier ampholytes in isoelectric fo-

cusing (16). Thus, the fact that NP-40, an uncharged ligand, can alter the pI of γ chains can only be interpreted by assuming that NP-40 either masks some charged groups on the polypeptide chains or alters the intrinsic pK of some ionizable groups in the neighborhood of the binding sites. The effect might be localized in the region of the mutation, since Ala is slightly more hydrophobic than Gly and thus could favor binding of NP-40 to the 133-141 residue stretch (which, in A_γ is: Met-Val-Thr-Ala-Val-Ala--Ser-Ala-Leu). By replacing Ala^{136} with Gly in G_γ the lower hydrophobicity of the Thr-Gly site might prevent binding of NP-40. The neighboring charged amino acids are Lys^{132}, Arg^{144} and His^{146}.

The number and relative activities of the different γ globin genes are still undetermined (1-7, 17); in particular, the recently discovered T_γ chain (7) has been suggested, on genetic grounds, to represent the product of an additional γ locus. The present technique, by allowing the preparation of purified G_γ and A_γ chains, has already made it possible to demonstrate that the T_γ chain is a variant A_γ globin (in preparation) and to measure directly the G_γ and A_γ relative synthetic activities in different erythroid populations.

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