Journal of Chromatography, 431 (1988) 177–183
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4278

Note

Reversed-phase high-performance liquid chromatographic separation and quantitation of reticulocyte α- and β-globin polypeptide chains from normal and β-thalassemic mice

MILLIE P. SCHAFER*

Laboratory of Molecular Hematology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892 (U.S.A.)

(First received January 8th, 1988; revised manuscript received May 2nd, 1988)

Hemoglobin production normally involves a balanced synthesis of α- and β-globin polypeptides resulting in the formation of tetramers composed of two α- and two β-subunits. Thalassemia is a genetic disease in which synthesis of α- and β-globin chains is abnormally low or absent. Recently, mouse models for human α- and β-thalassemias have been developed [1–3]. Examination of the α-globin chains relative to β-globin chains in the α-thalassemic mice indicated that the α-globin chains were present at 80% of normal levels instead of the expected 50% due to the loss of one of the two α-globin genes [2]. Thus, thalassemic mouse erythroid cells appear to compensate partially for the loss of half of their α-globin genes.

In β-thalassemia, there is a loss or deficiency of β-globin polypeptides, resulting in an excess of α-globin chains which form insoluble complexes with the developing red cells leading to ineffective erythropoiesis and hemolytic anemia [4]. In vitro examination of a mutation that produced an absolute deficiency of normal β-major globin polypeptides in a DBA/2J β-thalassemic mouse indicated that a compensatory effect occurred. An analysis of globin chain synthesis via [^{3}H]leucine incorporation revealed that β-globin synthesis was at least 75% of normal in the β-thalassemic mouse. Adult hemoglobin in this particular mouse strain is normally 80% $\alpha_2\beta_2^{\text{major}}$ and 20% $\alpha_2\beta_2^{\text{minor}}$. Theoretically, the β-thalas-

*Address for correspondence: Department of Microbiology and Molecular Genetics, Mail Location 524, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0524, U.S.A.

0378-4347/88/$03.50

semic mouse recovered from this strain was expected to only have a β^{minor}/α-globin ratio of 0.2 or 20% [1].

In order to analyze the production of globin polypeptide chains, all previous studies employed carboxymethyl-cellulose column chromatography using an 8 *M* urea–sodium phosphate buffer [5]. The resolution of the system was sufficient to enable the separation of α- and β-globin chains from several species and permitted a quantitative analysis of α- to β-globin ratios [6,7]. However, approximately 16–24 h was required for a single analysis, severely limiting an investigation into the mechanism of the compensatory increases in globin polypeptide chain formation. In addition, the carboxymethyl-cellulose column chromatography method did not resolve the two distinct, but highly homologous α-globin chains in the β-thalassemic mouse [8].

In this report, a rapid method, which can be utilized with both analytical (μg) and preparative (mg) procedures, for the separation and analysis of the mouse globin chains is presented. Mouse α- and β-globins differ from human and bovine α- and β-globin polypeptide chains, which have been resolved by various high-performance liquid chromatography (HPLC) methods [9–11]. The method also permits a rapid determination of which functional globin gene products are retained in thalassemic mice since the two α- and β-globins can be separated and identified by their position on the chromatogram. Previous analyses of various mouse strains in some cases required extensive protein sequencing [12] in order to determine which globin polypeptides were expressed.

EXPERIMENTAL

Erythroid cell preparation

Reticulocytosis was induced by multiple injections of phenylhydrazine as described [7]. DBA/2J mice obtained from Jackson Lab. were employed as controls. To collect the reticulocytes, retro-orbital bleeding was conducted.

In vitro translation of globin mRNA

Reticulocytes from phenylhydrazine-treated thalassemic and DBA/2J mice were collected and lysed in an equal volume of distilled water. To 35 μl of the lysate, potassium acetate, magnesium acetate, hemin, creatine phosphate, amino acids minus leucine and [^{3}H]leucine were added as described [7], in a final volume of 50 μl. The radiolabelled globin proteins were precipitated with acidic acetone at $-70\,^{\circ}$C for 10 min, lyophilized and resuspended in distilled water.

Reversed-phase high-performance liquid chromatography

Approximately 50 μg of protein were loaded onto a Beckman Ultrapore RPSC (Beckman Instruments, Berkeley, CA, U.S.A.) column (75 mm $\times$ 4.6 mm I.D.) equilibrated with 35 or 36% acetonitrile in 0.1% trifluoracetic acid. Chromatography was performed with a Waters HPLC system equipped with two Model 6000A pumps, a Model 720 system controller, and a 710 WISP for automatic sample injection. Absorbance was monitored at 280 nm with a Waters 440 detector. Globin chains were resolved with a 15-min linear gradient of 35–44% aceto-

nitrile in 0.1% trifluoroacetic acid or with a 20-min non-linear gradient of 36–51% acetonitrile (Waters curve 08) in 0.1% trifluoroacetic acid at a flow-rate of 1.0 ml/min. Fractions (0.125 ml) were collected directly into scintillation vials using an LKB2111 fraction collector. Incorporation of [^{3}H]leucine into globin chains was determined by the addition of 10 ml of Hydrofluor (National Diagnostics) to each vial, followed by liquid scintillation counting.

Amino acid analysis

Aliquots of each HPLC-purified globin polypeptide were lyophilized and resuspended in 6 *M* hydrochloric acid. The tubes were then evacuated and sealed, and the contents were hydrolyzed at 100°C for 20 h. Picomole level amino acid composition analysis was achieved via the ninhydrin reaction in a Beckman 6300 system essentially by the method of Spackman et al. [13].

RESULTS AND DISCUSSION

Reversed-phase HPLC analyses of the globin polypeptide chains from normal and thalassemic mice are shown in Fig. 1. Complete separation of the α- and β-globin chains is achieved in less than 15 min, in contrast to conventional carboxymethyl-cellulose column chromatography, which usually requires a minimum of 16 h. Amino acid composition analysis (Table I) indicates that a doublet peak representing two distinct α-globins eluted first from the column followed by the β-globin polypeptide chains. When the mouse globin proteins are loaded onto a carboxymethyl-cellulose column, the reverse occurs, the β-globins elute first followed by the elution of the α-globins as a single peak [8]. In Fig. 1A, the β-globin

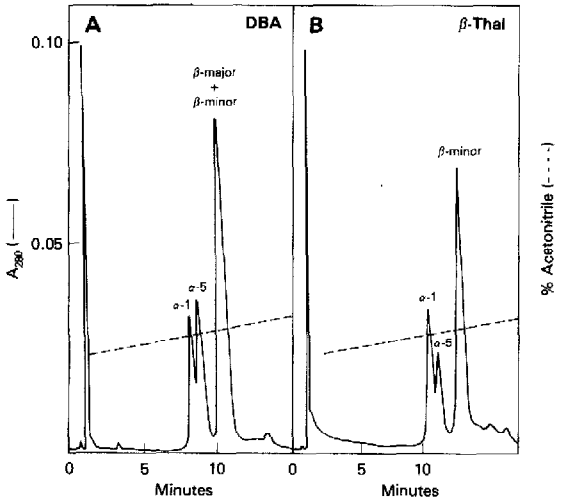

Fig. 1. Reversed-phase HPLC profile of mouse globin chains from an in vitro protein translation reaction. (A) DBA/2J normal parental strain chromatogram, (B) β-thalassemic mouse profile. The starting buffer contained 35% acetonitrile in 0.1% trifluoroacetic acid (TFA) with a 1.0 ml/min flow-rate using Waters program 06 (linear) gradient profile.

TABLE I

AMINO ACID COMPOSITION OF α-GLOBIN CHAIN 1 AND 5 AND β^{minor} GLOBIN POLYPEPTIDES FROM THE HOMOZYGOUS THALASSEMIC MOUSE

N.D. = not determined.

Amino acid	Predicted α* (residues/mol)	HPLC peak 1 α-1 (residues/mol)	HPLC peak 2 α-5 (residues/mol)	Predicted** β^{minor} (residues/mol)	HPLC peak 3 β^{minor} (residues/mol)
Ala	20	20	21	18	18
Arg	3	3	3	3	3
Asx	11	12	12	14	14
Cys	1	N.D.	N.D.	2	N.D.
Glx	5	5	5	9	9
Gly	11	11	10	12	12
His	11	11	11	8	8
Ile	2	2	2	4	4
Leu	17	17	17	17	17
Lys	11	10	11	12	11
Met	1	1	1	1	1
Phe	7	7	7	7	7
Pro	6	6	6	5	6
Ser	14	11	11	8	7
Thr	7	7	7	6	6
Trp	1	N.D.	N.D.	N.D.	N.D.
Tyr	3	3	3	3	3
Val	10	10	10	14	13

*From refs. 12 and 20. These values are from a BALB/c mouse strain which is highly homologous but not identical to the α-globins of the β-thalassemic mouse.
**From ref. 21.

peak from the normal mouse (DBA/2J) is actually a combination of two distinct β-globins referred to as β^{major} globin, representing 80% of the total amount of β-globin. The remaining 20% represents the β^{minor} globin component [14,15]. In Fig. 1B, the β-globin peak from the thalassemic mouse is only composed of β^{minor} globin since previous studies have shown that this particular mouse is completely deficient in β^{major} globin [1]. This is confirmed by the amino acid composition analysis presented in Table I.

To date, HPLC resolution of the two distinct, yet highly homologous α-globin chains had not been achieved. However, partial resolution was observed while separating α- from β-globins (Fig. 1). Further manipulation of the gradient system was then undertaken, and a complete separation of the α-globin chains was achieved (Fig. 2). The α-globin elution profiles for the normal (Fig. 2A) and the β-thalassemic mouse (Fig. 2B) were comparable, although concentration differences were observed.

Each α-globin peak was subject to total amino acid composition analysis. Due to the high degree of homology between HPLC peak 1 and HPLC peak 2 α-globins (Fig. 2), only two small differences were detected. The α-globin from peak 1 yielded one extra glycine and one less alanine when compared to peak 2. Previous studies, using protein sequence analysis, had shown that some inbred

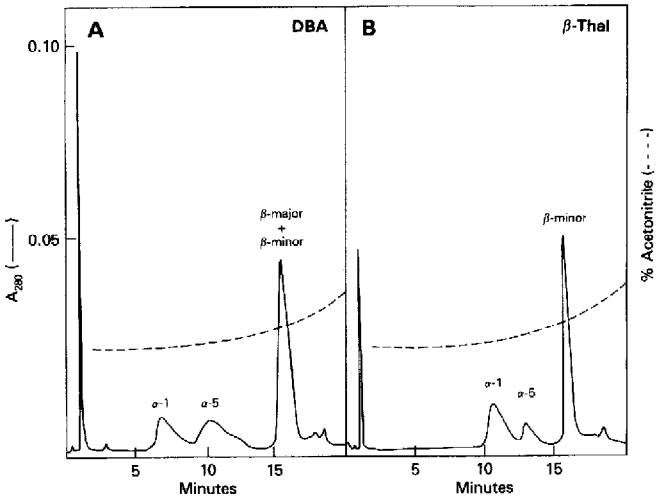

Fig. 2. Reversed-phase HPLC resolution of mouse α-globin chains from an in vitro protein translation reaction. (A) DBA/2J normal parental strain chromatogram, (B) β-thalassemic mouse profile. The starting buffer contained 36% acetonitrile in 01% TFA with a 1.0 ml/min flow-rate using Waters program 08 (non-linear) gradient profile.

strains of mice have two structurally distinct, highly homologous adult α-globins. For example, the BALB/c mouse strain has two α-globin forms differing only by a single amino acid. One form contained a serine, the other, a threonine at position 68 [12]. The β-thalassemic mouse as well as its DBA/2J parent each clearly have two distinct α-globins (Fig. 2). The β-thalassemic mouse was discovered as a spontaneous mutation among F1 progeny from matings of C57BL/6J female and a mutant DBA/2J male [1]. Using protein sequence analysis and isoelectric focusing, other investigators have demonstrated that the DBA/2J mouse expresses two kinds of α-globins, referred to as chain 1 and 5, while the C57BL/6J strain expresses a single type of α-globin chain, referred to as chain 1. Chain 1 contains a glycine at position 78 while chain 5 contains an alanine at this position [16,17]. The data from Fig. 2B and Table I indicate that the β-thalassemic mouse is expressing chain 1 and 5, but the ratio of these two chains is significantly different when compared to the normal mouse. The β-thalassemic mouse, heterozygous at all loci for different alleles compared to its homozygous inbred parental strains [18], expresses approximately twice the amount of chain 1 α-globin protein compared to chain 5 α-globin. It should be noted that the values presented here were derived from pooled samples of blood, i.e. blood from at least six mice were combined for each HPLC analysis. In addition, the α-globin haplotype for the β-thalassemic mice employed in these experiments were not genetically determined. So it is not known if individual differences among the pooled mice exist. Individual variants derived from other DBA/2J $\times$ C57BL/6J crosses have been detected. These basically appear to fall into three groups: group 1 express α-globin chain 1; group 2 express α-globin chain 1 and 5 either in equal amounts, or, α-globin chain 1 slightly exceeds the concentration of α-globin chain 5; group

3 also express α-globin chain 1 and 5, but in a 3:1 ratio [19]. Thus, the α-globin ratios presented here for the β-thalassemic mice reflect either an average of individual differences or an α-globin haplotype with a 2:1 ratio. Its normal parent (DBA/2J), as expected [17], expresses similar quantities of α-globin chain 1 and 5 (Fig. 2A) Thus, this approach will now permit the rapid, quantitative analysis of chain 1 and chain 5 α-globins expressed in other mouse strains as well as the progeny derived from crosses of these various inbred strains.

Reversed-phase HPLC of the mouse globin chains was initially undertaken in order to develop a rapid, quantitative method for studying the concentration of β-globin protein compared to the level of α-globin (β/α-globin chain ratios). This method would allow insight into the efficiency of translation of different globin mRNAs in thalassemic mice, a possible explanation for the higher amount of β^{minor} globin present in thalassemic mice when compared to normal mice. The compensatory mechanism which results in an elevated level of β^{minor} globin protein chains could occur at the translation level since studies in this laboratory have demonstrated little change in the β/α-globin mRNA ratios [7] Thus in vitro translation assays containing [^{3}H]leucine, followed by HPLC (Fig. 1) were undertaken using reticulocyte lysates from normal and β-thalassemic mice to confirm previously reported β/α globin ratios by other investigators using carboxymethyl-cellulose column chromatography [1]. Reticulocytes from control mice, DBA/2J, synthesized globin in a β/α ratio of 1.05 compared to the 1.06 previously reported value [1] The reticulocyte β^{minor}/α globin protein ratio from the β-thalassemic mouse was found to be 0.76 compared to the 0.78 value reported by Skow et al. [1]. These results are summarized in Table II. Studies were then undertaken to manipulate the β^{minor}/α ratio of the β-thalassemic mouse using cycloheximide as well as other reagents known to effet protein synthesis, demonstrating that the efficiency of translation of various globin mRNAs is differentially effected by these exogenous components as well as the endogenous components present in the reticulocyte lysate [7].

In summary, reversed-phase HPLC of the mouse globins permits a sensitive, rapid, quantitative analysis of α- and β-globin polypeptide chains in normal and thalassemic animals. Since both α-globin [2] and β-globin [1] thalassemic mice appear to compensate for the loss of globin proteins by an increase in α- and β-globin chain synthesis of the corresponding normal globin proteins, this tech-

TABLE II

β/α GLOBIN RATIOS FROM NORMAL AND THALASSEMIC MICE

Mouse strain	β/α Globin ratio	Previously reported value*
DBA/2J (control)	1.05	1.06
β-Thal (homozygous)	0.76	0.78
β-Thal (heterozygous)	–	0.92
		0.98

*From ref. 1.

nique may provide additional insights into protein translational regulatory mechanisms and permit a better understanding of the comparable human thalassemias.

ACKNOWLEDGEMENTS

John Owens provided the computerized BALB/c globin amino acid composition data. Judy DiPietro conducted the retro-orbital bleeding of the mice. Joan Curcio assisted Angela Murphy with the α- and β-globin amino acid composition analyses and ran the in vitro translation assays. In addition, a special thanks is extended to W. French Anderson, John A. Thompson, Ray A. Popp and Judy DiPietro for their helpful suggestions and critical review of this manuscript.

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