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GLOBIN CHAIN SEPARATION BY SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

SIMPLE SCREENING METHOD FOR ELONGATED HEMOGLOBIN CHAINS

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SUMMARY

A simple method for the separation of hemoglobin chains from hemolysate or globin, by sodium dodecyl sulfate polyacrylamide gel electrophoresis, is described. The α, β, and γ chains can be clearly separated from each other. The α chain has the highest mobility, the β chain has a slower mobility than the γ chain, while the δ chain has about the same mobility as the β chain. Hemoglobins with elongated chains can easily be detected by this method. Tak-β, elongated by 11 residues, moves much more slowly than βA but is much faster than α Constant Spring which is elongated by 31 residues. Screening of several individuals with slow-moving hemoglobins using this method led to the finding of a case with Hb Tak-β thalassemia and other carriers of Hb Tak.

INTRODUCTION

A number of methods for the electrophoretic separation of hemoglobin chains have been reported[1–5]. Separation of chains in polyacrylamide gel electrophoresis using acid or alkaline buffers was achieved by Moss and Ingram[3] and Stegink *et al.*[4]. Most of the methods described required globin for the study. The cellulose acetate electrophoretic method of Ueda and Schneider[5], which uses an alkaline buffer, and the acrylamide gel electrophoretic method described by Stegink *et al.*[4], which uses an acid buffer, can be performed using hemolysates. So far, in all the reported techniques, chain separation was achieved as a result of differences in the charge of the chains.

In this paper a new, simple method for separating hemoglobin chains, based on differences in molecular weight rather than on differences in charge, is described. The method employs sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis in which globin as well as hemolysates can be studied directly. In the presence of 2-mercaptoethanol, the anionic detergent SDS has the property to dissociate molecular structures to their ultimate polypeptide chains by breaking their disulfide bonds. These

polypeptide chains attached to SDS migrate in polyacrylamide gel electrophoresis according to their size rather than their electric charge.

MATERIAL AND METHODS

Hemolysates were prepared using toluene or carbon tetrachloride. Purified hemoglobin (Hb) components were obtained by the method of Huisman and Dozy[6] or by the inverted bottle method of Lie-Injo[7]. The latter is useful for the isolation of components that move very near to each other on starch-gel electrophoresis and column chromatography. The concentration of hemoglobin used can vary greatly, but it should be dilute in order to get a good separation of globin chains. Globin was prepared by acid–acetone precipitation.

Sample preparation

A sample of 10 μl of a 0.3–0.5 g % Hb (10–15 g % Hb solution diluted 30 times) or 10 μl of a 3–5 mg/ml globin solution in water is mixed with 0.2 ml of a buffer containing 0.1 ml mercaptoethanol, 0.2 ml of 0.5 *M* sodium phosphate pH 7.0, and 8.7 ml distilled water. Subsequently, 20 μl of 10% SDS is added (when preparing globin, it is important to add SDS last so that globin does not precipitate, but this sequence is not essential for hemolysate). The mixture is boiled for 2 min in a test tube placed in a beaker of boiling water. For each run 25 μl of this sample mixture is loaded onto the gel after mixing with 2 μl bromophenol blue, 1 μl mercaptoethanol and 5 μl of 50% glycerol.

Electrophoresis

The system used for electrophoresis is that of Laemmli[8] and O'Farrell[9] with slight modifications.

Polyacrylamide gel (20%) is prepared as follows: 30 ml of a stock solution of 30% polyacrylamide (29.2 g pure acrylamide and 0.8 g pure bis-acrylamide made up to 100 ml with distilled water) is mixed with 3.8 ml 10% glycerol, and 11.2 ml Tris buffer (containing 1.5 *M* Tris and 0.4% SDS), pH 8.8. Just prior to preparation of the gel, 0.1 ml of 10% ammonium persulfate and 20 μl of N,N,N′,N′-tetramethylenediamine (TEMED) are added. The mixture is poured into the slab gel electrophoretic cell. Either 0.75 mm or 1.5 mm gel thickness is preferred because the thin slab gel can be stained faster and be more easily dried at room temperature. The amount of gel solution prepared can be increased or decreased depending upon the size and thickness of the slab gel electrophoretic cell used.

Polyacrylamide gel (30%), prepared by using a 45% stock solution instead of 30% stock solution is suitable if run for a longer time.

Gradient (15–20%) SDS polyacrylamide gel electrophoresis also gives very good results but is more cumbersome.

Tris–glycine buffer (pH 8.3) 0.025 *M* Tris, 0.192 *M* glycine and 0.1% SDS (6.06 g Tris, 28.8 g glycine and 2 g SDS made up to 2 l with distilled water), is used in both upper and bottom tanks.

After a pre-run of 1–2 h, the samples are loaded. At the start of the run 5 V/cm are applied, and increased to 35 V/cm after 1 h. A good separation of globin chains can be achieved after about 5 h run, in the 20% gel. An overnight run in 20% gel

(Bio-Rad Model 221) gives more separation at the expense of the sharpness of the bands. There should be sufficient cooling (Lauda K-2R circulator) to prevent heating of the gel. Electrophoresis in a 30% gel is run overnight since mobility in this higher gel concentration is much lower. The higher gel concentration is preferred because it gives sharper bands.

Staining

After the run the gel is stained in 0.1% Coomassie blue in methanol–acetic acid solution (0.33 g Coomassie blue, 150 ml methanol, 150 ml water and 30 ml glacial acetic acid) for at least 4 h. After staining, the gel is washed repeatedly in a solution of methyl alcohol–water–glacial acetic acid in the proportions 10:10:1.

If desired, the gel can be dried by placing it between two sheets of cellulose (Bio-Rad) leaving free rims of cellulose on all sides. It is placed on a glass plate (the free cellulose edges being clamped against the plate) and dried at room temperature. Quantitation of the chains can be achieved by densitometric scanning of the stained bands.

RESULTS AND DISCUSSION

The human hemoglobin molecule consists of four polypeptide chains (usually two pairs of identical chains) to each of which a heme group is attached. Due to the action of SDS, these polypeptide chains dissociate and move in polyacrylamide-gel electrophoresis according to their size or molecular weight. This dissociation of the polypeptide chains apparently occurs in globin from which the heme groups have been stripped, as well as in whole hemoglobin in hemolysates. The free heme groups, which migrate independently, were not stained by the protein stain and were therefore not seen in the gel.

The α and β chains of normal Hb A are clearly separated by this method as are the α and γ chains of Hb F (Fig. 1). Mixed cord and adult hemolysates showed three bands, α, γ and β chains, clearly separated. The α chain moves fastest, as expected, since it has only 141 amino acid residues. Although β and γ chains both have

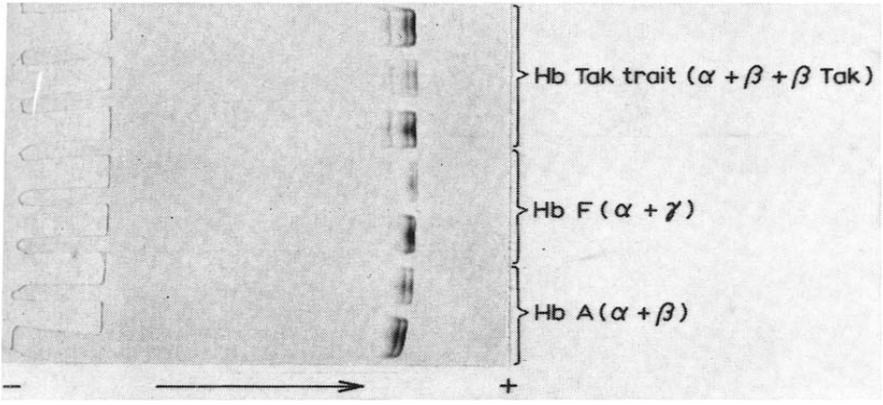

Fig. 1. Gradient SDS polyacrylamide gel (15–20%) electrophoresis. Tris–glycine buffer, pH 8.3. Separation of different globin chains according to molecular weight (35 V/cm, 3 1/2 h run).

146 residues, they can easily be differentiated by this method because the γ chain moves slightly faster than the β chain, apparently due to the difference in amino acid composition which results in a difference in size and molecular weight between the two chains. Hb Bart's (γ 4) from homozygous α thalassemia hydrops fetalis moves as a single band with the mobility expected of γ chains. The β chain of Hb S has glutamic acid replaced by valine at position 6. The difference in molecular weight between βA and βS, which is based upon the difference between the molecular weight of glutamic acid and that of valine, seems too small to be reflected in this method. The δ chain also has the same mobility as the β chain.

Elongated chains, such as the β chain of Hb Tak, which is elongated by 11 residues at the C terminus[10], and Hb Constant Spring (Hb CoSp) elongated by 31 residues[11], can easily be detected (Fig. 2). Purified Hb CoSp showed multiple slow-moving components (Fig. 2) which apparently differ in molecular weight. This is in agreement with the finding that it consists of chains of different lengths because of shortening, probably due to proteolysis[11]. Theoretically, shortened globin chains due to delections may also be detected by this method. Hb Leiden has glutamic acid residue deleted at position 6 or 7 of the β chain[12]; however, no clear difference in mobility can be detected between βA chains and βLeiden chains.

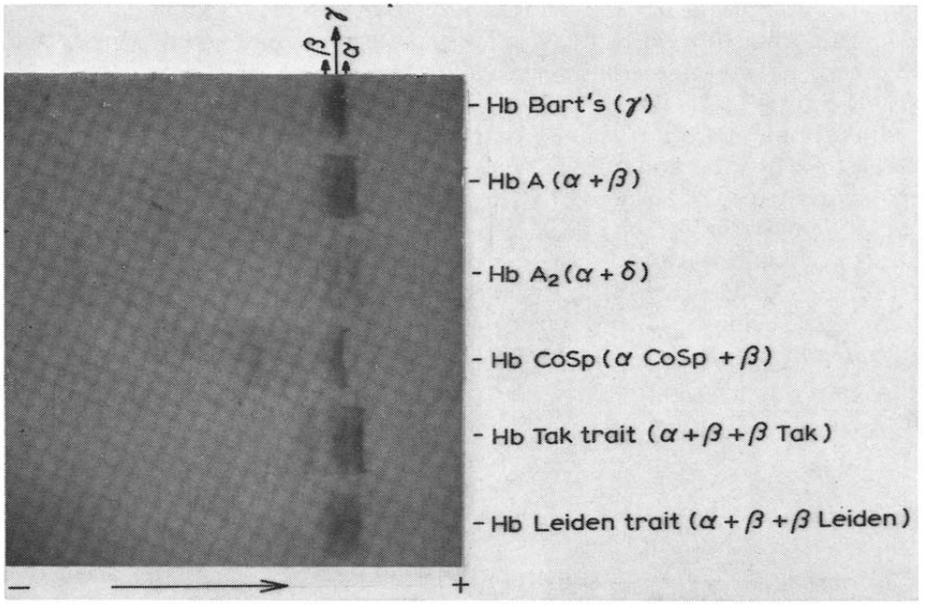

Fig. 2. SDS polyacrylamide gel (20%) electrophoresis. Tris–glycine buffer, pH 8.3, showing elongated globin chains β Tak and α CoSp. Globin chain β Leiden deleted by one residue shows the same mobility as β A (35 V/cm, 4 1/2 h run).

This method offers the advantage of giving good results with globin as well as with hemolysates. Because the technique is based on differences of molecular weight instead of on differences in charge, it may detect abnormal hemoglobins which could have been over-looked previously. It may be helpful to use this method to screen the

hemolysate of patients with hemolytic anemia resembling a hemoglobinopathy, which gives a normal electrophoretic pattern by the usual method. It also provides an additional procedure for the differentiation of slow-moving hemoglobins other than Hb S. Using this method for screening of several families known to have an abnormal hemoglobin which moved between Hb A and Hb A_2 but was found not to be Hb S, led to the discovery of a case of Hb Tak-β thalassemia and carriers of Hb Tak in the mother and several siblings (Fig. 3). This family will be described elsewhere.

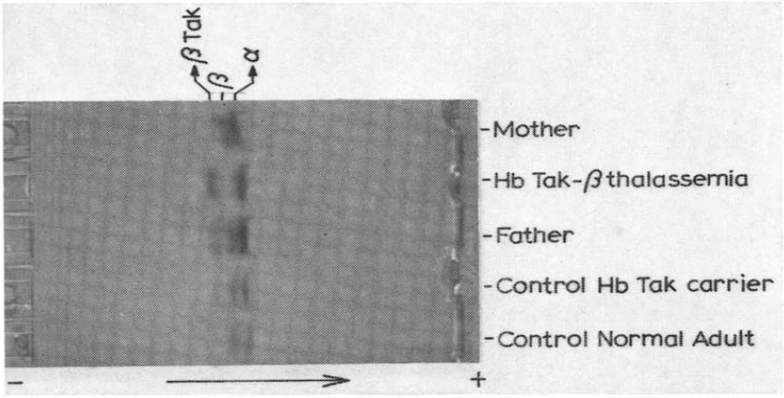

Fig. 3. SDS polyacrylamide gel (30%) electrophoresis. Tris–glycine buffer, pH 8.3, showing globin chain separation in hemolysates of a patient with Hb Tak-β thalassemia and of his parents (35 V/cm, 16-h run).

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