A Simple Method for Detection of Chain Anomaly of Abnormal Hemoglobin in Hemolysate without Preliminary Purification*

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About a decade ago electrophoresis was considered almighty for the identification as well as for the detection of abnormal hemoglobins. Hemoglobins A, S, C, D, E, and so forth were discriminated and identified by the difference in electrophoretic migration[1]. However, it soon became apparent that the molecule of hemoglobin was composed of two pairs of polypeptides, namely the α and the β chains, and that its abnormality in electrophoretic mobility mirrored the abnormal electric charge of its aberrant chains (or subunits), either α or β, which was directly related to the amino acid substitution in the relevant peptide[2]. Accordingly, establishment of the amino acid substitution has become mandatory for the identification of a newly discovered hemoglobin.

Usually, this used to be achieved through several steps including fingerprinting and amino acid analysis[3,4], and its first step was taken by the examination of the abnormal chain. Hybridization test of hemoglobins[5,6] and urea dissociation paper (or starch gel) electrophoresis of globins[7,8,9] have been used for this purpose during the recent several years. These methods are reliable, but both of them require preliminary purification of abnormal hemoglobin from the hemolysate. The possibility of determining the aberrant chain in the abnormal hemoglobin by direct examination of the hemolysate without purification[9] has seldom been conceived by the students of hemoglobin. However, it would be really convenient, if we could obtain a direct method for the detection of aberrant chain of the abnormal hemoglobin without need of its preliminary purification.

For the past several months we have been studying the dissociation of hemoglobin into its α and β subunits under the influence of p-chloromercuribenzoic acid, which was discovered by Bucci and his associates[10], in the hope that this may afford us a clue to the discovery of direct method for determining aberrant chain. The study has proved to be fruitful recently. We could successfully know the aberrant chain of the abnormal hemoglobin when we mixed the hemolysate containing both abnormal and normal hemoglobins with p-chloromercuribenzoic

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acid and analyzed it by starch gel electrophoresis.

The purpose of this paper is to present our new simple method for the determination of aberrant polypeptide chain by direct use of hemolysate as material.

MATERIAL AND METHOD

I. Materials : — Hemolysates (hemoglobin concentration, about 10g/dl) were prepared from the blood samples of normal subjects and of the patients with hemoglobinopathies by the conventional method.[11]

The hemolysates were examined by agar gel electrophoresis (pH 7.0 and 8.6)[12] to verify the presence of normal and abnormal hemoglobins.

The abnormal hemoglobins contained in these hemolysates had already been investigated by hybridazation test with canine hemoglobin,[6] urea dissociation paper electrophoresis of globins[7] and fingerprinting[3] before they were used for the present experiment. The hemoglobins were the following five variants: Hb Agenogi ($\alpha_2{}^A \beta_2{}^{Tp-10}$; slow-moving),[13] Hb Hiroshima ($\alpha_2{}^A \beta_2{}^{Tp14\sim15}$; fast-moving),[13] Hb Matsue ($\alpha_2{}^{Tp-9}\ \beta_2{}^A$; slow-moving),[14] Hb Shimonoseki ($\alpha_2{}^{54Arg}\ \beta_2{}^A$; slow-moving),[15] and Hb Ube-2 ($\alpha_2{}^{Tp-9}\ \beta_2{}^A$; fast-moving).[14]

II. PCMB method for the demonstration of chain anomaly.

1) Reagents.

(a) PCMB (4.2 mg/dl) solution : — An amount of 4.2 mg of p-chloromercuribenzoic acid is dissolved in 0.25 ml of 0.25 N aqueous sodium hydroxide solution, neutralized by adding 5 per cent acetic acid until trace amount of precipitate is formed, and then water is added to make the total volume of 1.0 ml.

(b) Aqueous sodium chloride (0.5 M) solution.

(c) Tris-citrate buffer solutin (pH 8.65): —Tris (hydroxymethyl) aminomethane (9.206 g) and citric acid C_3H_4 (OH) $(COOH)_3$ H_2O (1.051 g) are dissolved in water and made to 1000 ml with water (Tris : 0.076 M; citrate 0.05 M). This is used for the preparation of starch gel.

(d) Borate buffer solution : — Boric acid (18.552 g) and sodium hydroxide (2.40 g) are dissolved in water. This is made to 1000 ml with water. The solution is used for filling the electrode vessels. pH 8.1.

(e) Starch gel : — Potato starch (300 g) is suspended in 600 ml of the acetone : concentrated hydrochloric acid (120 : 1 v/v) mixture, allowed to stand at 37°C for 75 minutes for hydrolysis. The hydrolysis is stopped by adding 150 ml of 1 M sodium acetate solution. Then the potato starch (1 volume) is washed with water (5 volumes), resuspended in water (10 volumes), and placed in a refrigerator overnight. The starch is repeatedly washed with water (5 volumes) until the supernatant no longer gives positive chloride reaction against silver nitrate solution. The supernatant is decanted, and the starch is dehydrated with acetone. Dry powder of hydrolyzed potato starch is prepared in this way.

Dry hydrolyzed potato starch (15 g) is suspended in 100 ml of tris-citrate buffer solution in a round-bottom flask. The suspension is heated over a burner until it becomes translucent and fluid. The flask is connected with vacuum pump to remove the air bubbles in the hot starch solution completely under reduced pressure. The starch solution is poured into an acrylite tray ($20 \times 6 \times 0.3$ cm) while it is hot, covered tightly with an acrylite plate, and stored at cool place for a day to be solidified adequately firm.

2) Procedure.

(a) PCMB treatment : — To 0.5 ml of hemolysate which was saturated with carbon monoxide to convert all the hemoglobins into carbonmonoxyhemoglobin are added 0.2 ml of 0.5 M sodium chloride solution and 0.5 ml of PCMB solution so that the ratio PCMB/HbCO becomes 8 M/M. The pH of the mixture is adjusted to 6.0 with dilute acetic acid, and its total volume is brought to 2.0 ml with water. The mixture is allowed to stand at 4°C overnight. The dissociation of hemoglobin into its α and β subunits can be adjusted as one desires by regulating the amount of sodium chloride added to the mixture or by changing the pH of the medium. The proportion of dissociated hemoglobin increases with increase in sodium chloride concentration or with the fall of pH.

(b) Starch gel electrophoresis : — At an adequate place in the starch gel a slit (about 10 mm in length) is cut with a razor blade. A small rectangular piece of filter paper (Whatman 3 MM, about 10×2.5 mm in size) is dipped into the PCMB-treated hemolysate, blotted and applied into the slit. The electrophoresis assembly is arranged (as described above). The starch gel tray is covered with a sheet of parafilm tightly, and placed horizontally. Its two ends are cennected with electrode vessels with filter paper wicks. An electric current (10 v/cm) is sent from a voltage stabilizer for four hours. At the end of the time the electric source is disconnected, and the starch gel is removed gently from the tray to be stained with amido-black[16] or benzidine[17] by conventional techniques.

RESULTS

When normal hemolysate was treated with PCMB and subjected to starch gel electrophoresis, there appeared three groups of electrophoretic stripes on the gel : (1) two stripes of roughly equal intensity migrating to the anode side, (2) a heavy stripe closely accompanied by a faint stripe moving to the cathode side, and (3) an indiscretely delineated stripes or bands lying in the intermadiate area. These are referred to as "anodal", "cathodal" and "intermediate" group, respectively.

Hb Agenogi which had been separated from Hb A and purified by starch block electrophoresis gave no stripes of "anodal" group, but, instead, yielded a new group of stripes (in a pair) lying close together which occupied the place between the "anodal" group and the "intermediate" group. This new group of stripes was

again clearly visible on starch gel when the hemolysate of Hb Agenogi hemoglobinopathy (content of abnormal hemoglobin was 50 per cent) was treated with PCMB similarly. However, in this case "anodal" group of stripes which was seen in the PCMB-treated normal hemolysate was also seen. Therefore, the hemolysate of Hb Agenogi hemoglobinopathy showed four groups of electrophoretic stripes: The "anodal", the "new", the "intermediate" and the "cathodal" lining up from the anode to the cathode in the order mentioned (Figure 1).

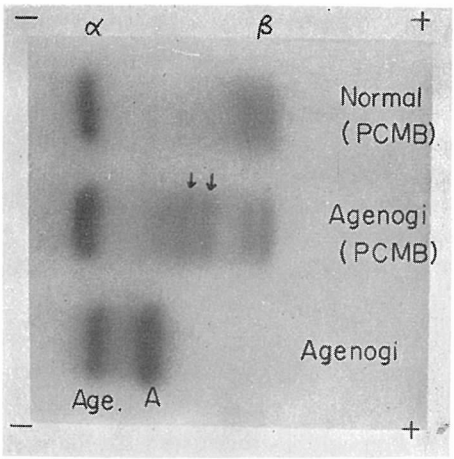

Fig. 1. Starch gel electrophoresis of Hb Agenogi hemolysate (before and after PCMB treatment) and PCMB-treated normal adult hemolysate as a control material. Hb Agenogi is a slow-movig hemoglobin and its "new" group of stripes representing the aberrant chain (indicated by arrows in the photograph) is positioned between the "anodal" (β chain) and "cathodal" (α chain) groups near the "initermediate" (undissociated Hb A) group. Therefore, it is presumed that Hb Agenogi is a slow-moving hemoglobin with β chain anomaly. A: Hb A. Age.: Hb Agenogi. α: α chain. β: β chain.

Similar electrophoretic patterns were observed also with the hemolysates of four other hemoglobinopathies, namely, in Hb Matsue hemoglobinopathy ("anodal", "intermediate", "cathodal" and "new"), in Hb Ube-2 hemoglobinopathy ("anodal", "intermediate", "new" and "cathodal"), in Hb Shimonoseki hemoglobinopathy ("anodal", "intermediate" "cathodal", and "new"), and in Hb Hiroshima hemoglobinopathy ("new", "anodal", "intermediate" and "cathodal").

DISCUSSION

It is apparant that the most important problem in our study is the interpretation of the "anodal", "intermediate" and "cathodal" groups of the electrophoretic stripes. To elucidate the nature of the stripes the following experiment was carried out.

Normal hemolysate was treated with PCMB to analyze it by carboxy-methyl cellulose chromatography.[10,19] Six fractions from the first to the sixth mentioned in the order of descending the column, were separated by this technique. The second and the fourth were abundant, constituting the major fractions, while other fractions were relatively small in amount, forming minor fractions. These fractions were collected individually, introduced into visking tubes (8/32) separately, and

concentrated by ultrafiltration technique which was invented by Hofsten and Falkbring.[18] The concentrates were examined by means of urea dissociation paper electrophoresis[7] and by fingerprinting (Baglioni)[4] after digestion with trypsin.

The results of this experiment demonstrated that the second and the fourth farctions were identical with β chain and α chain, respectively. Urea dissociation paper electrophoresis of the concentrate of the third fraction disclosed that it was undissociated Hb A which was composed of equivalent amounts of α and β chains.

The second, the third and the fourth fractions (which had been concentrated) were subjected to starch gel electrophoresis (pH 8.65) along with the PCMB-treated normal hemolysate. The electrophoretic patteern revealed that the second fraction (β chain), the third fraction (undeissociated Hb A), and the fourth fraction (α chain) were equal to the "anodal", "intermediate" and "cathodal" groups, respectively, in migration. It was accordingly presumed that the "anodal" group represented the β chain, the "intermediate" group the undissociated hemoglobin A, and the "cathodal" group the α chain. The presumption is in complete agreement with the conception which was entertained by Bucci and his associates[10,19] who emplyed carboxymethyl cellulose chromatography and fingerprinting of the tryptic digests in combination for studying the nature of the electrophoretic stripes of the PCMB treated hemolysate.

Theoretically, the electric charge of the hemoglobin molecule depends on the electric charges of its ingredients, namely, of the α and β chains. If an abnormal hemoglobin has aberrant α or β chains which are more negatively charged than those of normal hemoglobin, the hemoglobin will be fast-moving electrophoretically. Conversely, if a particular abnormal hemoglobin is with aberrant α or β chains which are smaller in negative charge than those of normal hemoglobin, it will be slow-moving. These aberrent α or β chains, either larger or smaller in negative charge, is expected to make their appearance as "new" group of stripes on starch gel because of their abnormal electric charge when a PCMB-treated hemolysate of hemoglobinopathy is examined electrophoretically. The "new" group of stripes will be easily recongnized as such, if the PCMB-treated hemolysate of hemoglobinopathy is subjected to electrophresis simultaneously with the same hemolysate without PCMB treatment and the PCMB-treated normal hemolysate on the same starch gel. Furthemore, theoretical contemplation will suggest four possible electrophoretic migration patterns of the "new" group of stripes in relation to those of the "anodal" (β) or the "cathodal" (α) group as shown in figure 2, and table 1.

It is interesting that this figure covers all the electrophoretic patterns of the "new" group of stripes of the hemolysates of hemoglobinopathies, which were actually studied and described in the previous section.

Scrutiny of the figure in comparison with table 1 will lead us to presume α chain anomaly for Hb Ube-2, Hb Matsue and Hb Shimonoseki and β chain anomaly for Hb Hiroshima and Hb Agenogi. The presumption is in complete agreement with the chain anomalies[13,14,15] which were already determined by

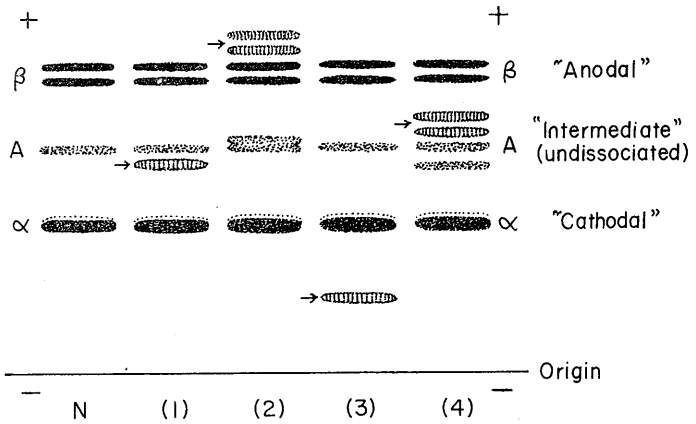

Fig. 2. Starch gel electrophoresis of PCMB-treated hemolysate of hemoglobinopathies. N. Normal adult hemolysate, (1) Hb Ube 2: fast-moving, α chain anomaly, (2) Hb Hiroshima: fast-moving, β chain anomaly, (3) Hb Matsue or Shimonoseki: slow-moving, α chain anomal y, (4) Hb Agenogi: slow-moving, β chain anomaly. The "new" group of stripes representing the aberrant chain is indicated by an arrow.

Table 1. Possible four migration patterns of the aberrant chains.

Abnormal hemoglobin	Aberrant chain	Aberrant chain
Fast-moving	(1) Between the "anodal" (β) and the "cathodal" (α)	(2) To the anode side of the "anodal" (β)
Slow-moving	(3) To the cathode side of the "cathodal" (α)	(4) Between the "anodal" (β) and the "cathodal" (α)

(1) Ube-2 (2) Hiroshima (3) Shimonoseki (4) Agenogi

hybridization test with canine hemoglobin, urea dissociation paper electrophoresis of globins and fingerinting. It is accordingly warranted to think that new method is reliable as a measure for the determination of abrrant chain in abnomal hemoglobins.

Starch gel electrophoresis of the PCMB-treated hemolysate is simple in procedure. It requires no preliminary purification of abnormal hemoglobins, which is mandatory for the performance of the conventional methods such a hybridization

test and urea dissociation paper electrophoresis. It saves time and labor for study. Hemolysate as small in amount as 0.1 ml is sufficient for the analysis. This test is recommended as a simple and rapid method for the detection of aberrant chain in the newly discovered abnormal hemoglobin.

SUMMARY

A new simple and rapid method for determining chain anomaly of abnormal hemoglobin was invented.

Hemolysate, without preliminary separation and purification of the abnormal hemoglobin, is treated with p-chloromercuribenzoic acid (PCMB) in acidic solution, and subjected to starch gel electrophoresis (pH 8.65). As control materials the hemolysate (of the same hemoglobinopathy) without PCMB treatment and the PCMB-treated normal hemolysate are applied to the same starch gel.

Three main groups of electrophoretic stripes appear in the PCMB-treated normal hemolysate: (1) the "anodal" representing β chain, (2) the "intermediate" referring to undissociated Hb A, and (3) the "cathodal" referring to α chain. In the PCMB-treated hemolysate of hemoglobinopathy another group of stripes, namely the "new", makes its appearance in addition to the three groups.

The chain anomaly of the abnormal hemoglobin is presumed by examining the electrophoretic migration of the "new" in comparison with those of the "anodal" and the "cathodal". The criteria of the presumption is summarized as follows.

If the relevant abnormal hemoglobin is fast-moving electrophoretically, the "new" possessing the migration intermediate of the "anobal" (β chain) and the "cathodal" (α chain) indicates α chain anomaly, and the "new" migrating more closely to the anode than the "anodal" (β chain) referrs to β chain anomaly.

If the abnormal hemoglobin is slow-moving, the "new" situating more closely to the cathode than the "cathodal" (α chain) is indicative of α chain anomaly, and the "new" migrating between the "anodal" (β chain) and the "cathodal" (α chain) is suggestive of β chain anomaly.

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