Artificial Synthesis and Hybridization of Hb M_{Iwate}*

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Hemoglobin M_{Iwate} (Hb M_{Iwate}) is a chocolate-colored abnormal hemoglobin which was discovered from the blood of a patient with hereditary nigremia by Shibata and his associates in 1960.[1]

Chemical studies[2,3] made during the past several years clarified that the four hemes which are arranged to the globin of Hb M_{Iwate} are identical with protoporphyrin-iron of the adult hemoglobin (Hb A). This abnormal hemoglobin has aberrant α chains (α^{M_I}) in which the 87th amino acid residue is Tyr that forms a stable phenolate complex with ferric heme iron.[4] Accordingly, the α^{M_I} subunits of Hb M_{Iwate} are neither oxygenated reversibly, nor reduced by DPNH-dependent diaphorese and sodium dithionite. Ligands such as fluoride, azide and hydrogen peroxide do not react with the α^{M_I} subunits, and even cyanide which readily converts met-Hb A into cyanmet-Hb A fails to alter them.[5] However, the β subunits of Hb M_{Iwate} are essentially normal, namely the same as the β^A chains of Hb A.

In other words, the molecule of oxy-Hb M_{Iwate} is composed of two different halves: (1) met-hemoglobin-like α^{M_I} subunits incapable of oxygen transportation, and (2) normally oxygenated β^A subunits. They are united as expressed in the formula: $\alpha_2^{M_I}\beta_2^A$.

This conception has recently been reconfirmed in our laboratory by the experiment of artificial reconstitution of Hb M_{Iwate} and by the test of hybridization of Hb M_{Iwate} with canine hemoglobin.

This communication describes the results of our heme exchange experiment and hybridization of Hb M_{Iwate}.

METHOD AND MATERIALS

A) Reconstitution of Hb M_{Iwate} and Hb A from heme and globin.

(1) Preparation of hemin: Eluate of pure met-Hb M_{Iwate} obtained by starch block electrophoresis (pH: 7.0)[6] of the met-Hb type hemolysate of hereditary nigremia was concentrated to approximately two gm per cent with Sephadex.

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To this concentrated eluate were added 50-fold volumes of ice cold acid acetone of Anson-Mirsky[7] and centrifuged. The supernatant acetone layer containing the hemin of Hb M_{Iwate} (M-hemin) was evaporated off under reduced pressure after a small amount of sodium chloride had been added. In this way crystals of M-hemin chloride were obtained at the bottom of the flask. They were collected and dissolved in pyridine chloroform mixture. Glacial acetic acid-HCl mixture was added to the solution to recrystallize M-hemin chloride.

Hemin of Hb A (A-hemin) was prepared from 120 ml. of blood of a normal subject by the method of Willstätter.[8] A-hemin chloride was dissolved and recrystallized two times with pyridine-chloroform and glacial acetic acid-HCl mixture as described above.

(2) Preparation of native globin: Globin of Hb M_{Iwate} (M-globin) was prepared from pure oxy-Hb M_{Iwate} which was obtained by agar gel electrophoresis. Oxy-Hb M_{Iwate} eluate from agar was concentrated to approximately one gm. per cent with Sephadex to get native M-globin solution by the method of Fanelli and Antonini.[9] The M-globin solution thus prepared was transparent and yellowish at pH 6.8 with a concentration of about one gm. per cent.

The same procedure was employed for the preparation of the solution of Hb A globin (A-globin). It was 6.0 in pH and about 0.6 gm. per cent in concentration.

(3) Coupling of hemin with globin: To 3 ml of globin solution was added 0.03 ml of saturated solution of hemin chloride in 0.02-NaOH and kept at 0°C for 15 to 20 minutes so that coupling might proceed without denaturation of the globin. The synthesis of hemoglobin was examined in a photoelectric spectrophotometer.

B) Hybridization of Hb M_{Iwate} with canine hemoglobin.

(1) Preparation of hemoglobin solution: Solution of purified met-Hb M_{Iwate} obtained by starch block electrophoresis (pH: 7.0) was introduced into a Visking tube and dialysed against 0.1 M phosphate buffer (pH: 6.8) in a refrigerator to equibrate pH. The dialysate (approximately one to two gm. per cent) was determined spectrophotometrically for its hemoglobin concentration by measuring the absorbance at 280 mμ. An amount of ascorbic acid five times as much as that theoretically required for complete reduction of Hb M_{Iwate} was dissolved in a minimum amount of 0.04 M phosphate buffer (pH: 6.8). This was added to the dialysate to convert its hemoglobin into reduced form, followed by dialysis against a large amount of 0.04 M phosphate buffer (pH 6.8) at 4°C for 24 hours. At the end of the time one half of the content in the Visking tube was exposed to carbon monoxide gas to get COHb M_{Iwate} solution. The remaining half was treated with a minute amount of sodium dithionite after its reaction had been adjusted to weak alkalinity (pH: 8.0). Then it was subjected to electrodialysis

against cold phosphate buffer (pH: 8.0) to get a solution of reduced Hb M_{Iwate} completely ridden of the reducing reagent, and finally shaken with air so that all the Hb M_{Iwate} might be oxygenated.

Both of the solution of CO Hb M_{Iwate} and O_2 Hb M_{Iwate} were used for hybridization.

(2) Hybridization. A 1 : 1 mixture of equimolar solution of Hb M_{Iwate} and canine hemoglobin (Hb Can) was introduced into a Visking tube to be dialysed against 0.1 M acetate buffer (pH 4.7) for 48 hours in a refrigerator (The hemoglobins were dissociated into their α and β chains). Then the Visking tube was transferred into 0.04 M phosphate buffer (pH: 6.8) and allowed to remain there for 24 hours (The α and β chains were recombined and hybrid hemoglobins were formed).[10] Insoluble brown precipitate was removed by centrifugation at 0°C. The supernatant was subjected to starch gel electrophoresis (pH: 6.8 and 8.6)[11] to demonstrate the recombined and the hybrid hemoglobins.

RESULTS

Coupling of hemin with globin was recognized by the gradual increase in red color attaining its maximum intensity 15 minutes after mixing. The synthesized hemoglobin remained stable for several hours even when the mixture was kept at room temperature. This made a sharp contrast to the native globin which produced precipitate within a few minutes.

The hemoglobin artificially synthesized with M-hemin and A-globin showed spectroscopically an absorbance which was disproportionately large to the sum of the absorbances of equimolar M-hemin and A-globin used for coupling. The hemoglobin was essentially the same as met-Hb A in the shape of absorption curve, possessing its absorption maximum at 500 mμ and 630 mμ. Addition of sodium dithionite immediately resulted in a remarkable change, ultimately giving rise to an absorption curve resembling that of reduced Hb A with a single large, symmetric absorption peak at 556 mμ and a small atypical protrusion around 630 mμ. (Figures 1 and 2).

When M-globin was coupled with A-hemin in neutral medium (pH: 6.8), the hemoglobin synthesized in the mixture was spectroscopically identical with neutral met-Hb M_{Iwate} which possessed its absorption maximum at 590 mμ. When they were coupled in alkaline media (pH: 8.6), alkaline met-Hb M_{Iwate} was obtained (Figures 3 and 4).

The M and A globins as well as the M and A hemins could not be discriminated by their absorption curves.

Starch gel electrophoresis of the recombinant mixtures of CO Hb M_{Iwate} (or O_2 Hb M_{Iwate}) and Hb Can revealed the presence of two hybrids in addition to the two original hemoglobins. They formed a mosaic pattern of two pairs of

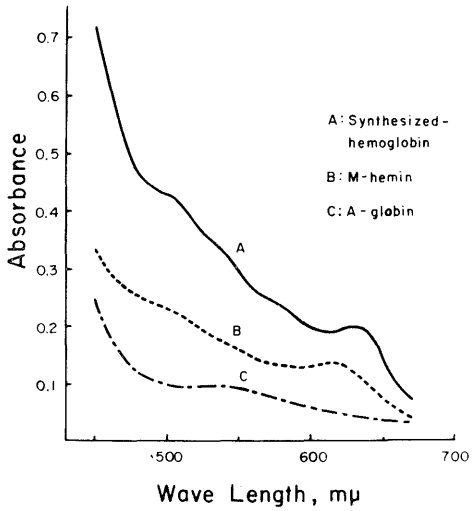

Figure 1. Synthesis of methemoglobin A with the hemin of Hb M_{Iwate} (M-hemin) And the globin of Hb A (A-globin).

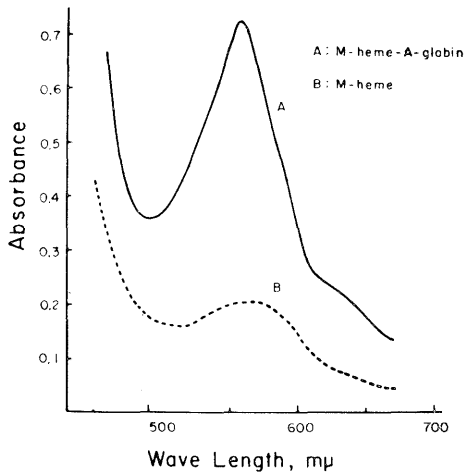

Figure 2. Addition of sodium dithionite to the artificially synthesized methemoglobin (Figure 1) yields reduced hemoglobin A (M-heme-A-globin). Heme of Hb M_{Iwate} (B: M-heme) is shown for the sake of comparison.

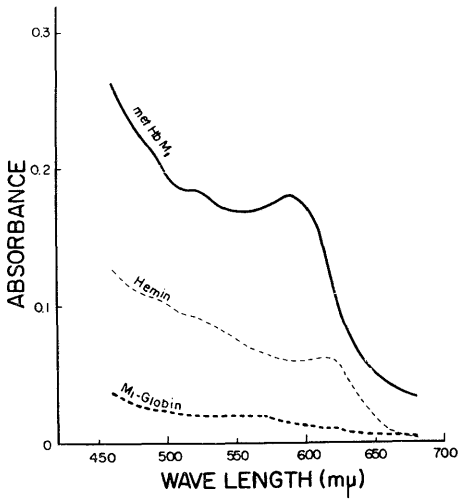

Figure 3. Methemoglobin M_{Iwate} (met Hb M_I) synthesized in neutral medium (pH 6.8) from the hemin of Hb A and the globin of Hb M_{Iwate} (M_I-globin).

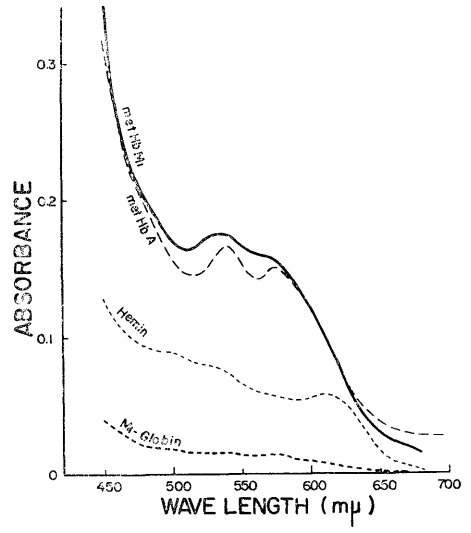

Figure 4. Methemoglobin M_I (met Hb M_I) synthesized in alkaline medium (pH 8.6) from the hemin of Hb A and the globin of Hb M_{Iwate} (M_I-globin).

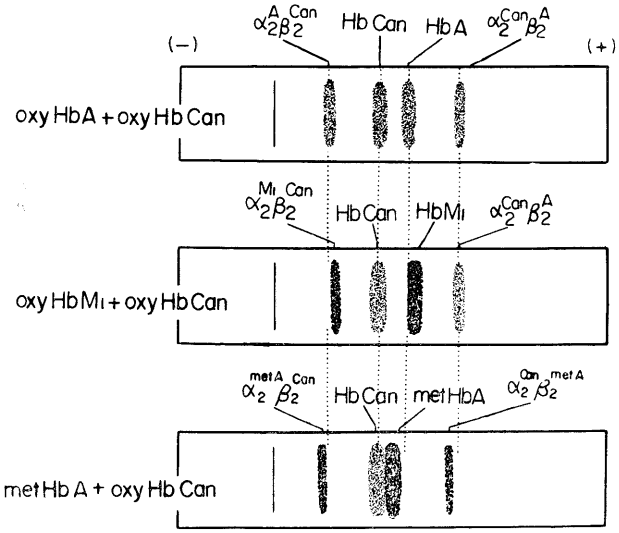

Figure 5. Schematic illustration of the result of starch gel electrophoresis of hybrid and original hemoglobins.
Finely dotted stripes and coarsely dotted stripes are red and black in color, respectively.

red and black stripes that were lined up from the anode side to the cathode side in the order of (1) red hybrid (α_2^{Can} β_2^{A}), (2) black hemoglobin (α_2^{MI} β_2^{A}), (3) red hemoglobin (α_2^{Can} β_2^{Can}), and (4) black hybrid (α_2^{MI} β_2^{Can}) (Figure 5).

The cathodal hybrid (α_2^{MI} β_2^{Can}) was black (chocolate-brown) visually, assuming the same color as that of Hb M_{Iwate} on starch gel. This chocolate colored portion of starch gel was cut out and buried in starch block (pH: 7.0). An electric current was sent through the starch block to move the black hybrid hemoglobin into the starch and its was eluted for the purpose of spectroscopy. There was a good agreement of the absorption curves between the eluate and neutral solution of met Hb M_{Iwate}. The hybrid at the anodal side (α_2^{Can} β_2^{A}) was red, being essentially the same in absorption as oxy-Hb A.

As a control test, formation of hybrid between met-Hb A and Hb Can was also examined. The cathodal hybrid (α_2^{metA} β_2^{Can}) and the anodal hybrid (α_2^{Can} β_2^{metA}) were brown in color. Difference spectroscopy of the absorption spectrum of the eluate of the cathodal hybrid disclosed that the hybrid was a combination of half-molecules of met Hb A and oxy Hb Can.

DISCUSSION

It is obvious from the fore-going account that typical acid met-Hb A is artificially synthesized by coupling M-hemin with A-globin. Absence of significant difference in the absorption spectrum between M-globin and A-globin indicates

that the hemes are successfully detached from the α^{M_I} chain of Hb M_{Iwate} by acidic acetone (Anson-Mirsky) in spite of stable phenolate complex formed between ferric heme iron and Tyr, the substitute of His (87). There is no doubt that in Hb M_{Iwate} both α^{M_I} and β^A subunits have the same hemes as those of Hb A. Artificial synthesis of hemoglobins has reconfirmed the normal character of hemes in Hb M_{Iwate} that was deduced in the previous communication[2] from the result of the spectroscopy of its pyridine hemochrome and alkali hemochrome.

Indeed, the absorption curve of the synthesized hemoglobin (reduced from) shows a slight protrusion around 630 mμ. However, this is not related to the abnormality of heme, but to the comtamination of slightly denatured globin which has been generated during the preparation of native globin. Similar protrusion was observed by Satake and his associates[12] when they reduced the reconstituted met-Hb A, met-Hb H and met Hb α with sodium dithionite.

Successful synthesis of met-Hb M_{Iwate} from M-globin and A-hemin furnishes a substantial evidence for the conception that the peculiar chocolate-brown color of Hb M_{Iwate} is the result of the abnormality of its globin.

In 1948, Hörlein and Weber[13] reported the first family of Hb M disease (chronic familial methemoglobinemia), which was at the same time the first pedigree of hemoglobinopathy that had ever been recorded in the world. They demonstrated the abnormality of globin by heme exchange experiment using a patient's hemolysate, which was not a pure Hb M solution, but a mixture of Hb A and Hb M in the light of the present knowledge of this disease. In this respect our experiment will be regarded as the one which substantiates their view with purified M-globin as material.

In the hybridization test, the hybrid ($\alpha_2^{M_I}$ β_2^{Can}) was almost the same in color as O_2 Hb M_{Iwate}. Its absorption spectrum was, however, of met-Hb M_{Iwate}. Presumably the β^{Can} subunit of the hybrid was oxidized to met-Hb form. In contrast, the hybrid (α_2^{MetA} β_2^{Can}) bore spectroscopically a close resemblance to the theoretically supposed combination of met-Hb A and oxy Hb Can in proportion of 1 : 1. Probably, the α^{M_I} subunits will render their partner β^A subunits auto-oxidized more easily than do the α^{MetA} subunits. Any-way, the formation of the black hybrid ($\alpha_2^{M_I}$ β_2^{Can}) is a conclusive evidence that α^{M_I} subunits are responsible for the chocolate-brown color of Hb M_{Iwate}.

CONCLUSION

Heme exchange between Hb M_{Iwate} and Hb A, and hybridization of Hb M_{Iwate} with canine hemoglobin were studied in order to elucidate the essential character of the color of this abnormal hemoglobin. Methemoglobins of Hb M_{Iwate} and Hb A were successfully reconstituted by coupling the hemin of Hb A with the globin of purified Hb M_{Iwate} and by uniting the hemin of Hb M_{Iwate} with the globin of Hb A, respectively. Hybridization of Hb M_{Iwate} with canine hemoglo

bin yielded a black cathodal hybrid (α_2^{MI} β_2^{Can}) and a red anodal hybrid (α_2^{Can} β_2^{A}) in addition to the original hemoglobins.

The result of experiments furnishes conclusive evidence that the chocolate-brown color of Hb M_{Iwate} is directly concerned with its abnormal globin possessing aberrant α chains.

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