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Note

Isolation of normal and variant human hemoglobin subunits

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Hemoglobin has always served as an ideal model for the study of allosteric systems [1]. Structural and functional characterization of human hemoglobin variants continues to provide a wealth of information about the molecular basis of cooperative ligand binding. Hemoglobin, a tetramer composed of two pairs of unlike subunits, can also be employed as a model protein for the study of macromolecular assembly. Understanding of this subunit assembly process of hemoglobin has been enhanced by our recent studies of selected hemoglobin variants [2—5]. During the course of these experiments it became evident that although traditional methods of chain purification [6—8] allowed successful preparation of native normal alpha and beta subunits, these available methods did not yield variant hemoglobin subunits that fully recombined into native hemoglobin tetramers. A newly developed protocol for the preparation of isolated hemoglobin subunits is reported here.

EXPERIMENTAL

Materials

The *p*-hydroxymercuribenzoate (PMB) obtained from Sigma (St. Louis, MO, U.S.A.), 2-mercaptoethanol from Aldrich (Milwaukee, WI, U.S.A.), and all additional chemicals from Fisher (Pittsburgh, PA, U.S.A.) were of the highest purity available and were used without further purification. Sephadex G-25 (Pharmacia, Piscataway, NJ, U.S.A.) was swollen in distilled water and then converted to the required pH and ionic strength with buffer. After removal of fines with distilled water, the CM-52 cellulose (Whatman, Clifton, NJ, U.S.A.) was initially precycled with 0.1 *M* sodium hydroxide followed by

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treatment with 0.1 *M* hydrochloric acid and subsequent equilibration with the appropriate buffer. DE-52 cellulose (Whatman), after being washed with 0.1 *M* hydrochloric acid and then 0.1 *M* sodium hydroxide, usually had to be titrated sparingly with concentrated phosphoric acid and then could be equilibrated with buffer. DEAE-Sephadex A-50 and QAE-Sephadex A-50 (Pharmacia) were equilibrated directly in buffer for several days at room temperature.

General methods

Isolated subunits of normal adult hemoglobin were obtained using the methods of Bucci and Fronticelli [6] as modified by Geraci et al. [7] and McDonald and Noble [8]. The purity of the isolated subunits and their ability to fully recombine were confirmed by cellulose acetate electrophoresis. Regeneration of the sulfhydryl groups was checked by PMB titration [8]. Oxygen equilibrium measurements were carried out to ensure that the reconstituted hemoglobin tetramer was a fully cooperative hemoglobin exhibiting normal oxygen affinity [9].

RESULTS AND DISCUSSION

Erythrocytes from normal individuals and from patients with hemoglobinopathies were washed three times with 0.15 *M* sodium chloride and either used immediately or frozen in liquid nitrogen until needed. The washed red blood cells were lysed with distilled water, followed by high speed centrifugation. After dialysis against distilled water, the hemoglobin was reacted with an excess of the sulfhydryl reagent, PMB. The PMB reacts with the protein sulfhydryl groups to split the hemoglobin molecule into its constituent alpha and beta heme subunits. The PMB derivative yielded excellent splitting (> 95%) for all normal and variant hemoglobins and was prepared in the following manner. PMB (50 mg) was dissolved in 1 ml of 0.1 *M* sodium hydroxide and back titrated with 1.0 *M* acetic acid immediately prior to use. This was reacted with 10 ml of a solution of Hb A (4.7–6.3 m*M* in heme) to which 0.1 ml of 1.0 *M* potassium monobasic phosphate and 0.4 ml of 2.0 *M* sodium chloride had been added. The pH of this PMB derivative was adjusted to 6.0 with 1.0 *M* acetic acid and the solution was left overnight (16 h) at 4°C. All subsequent steps in purification were carried out at this temperature. The flocculent precipitate which appeared was readily removed by centrifugation. Excellent splitting of the parent beta variant hemoglobins [Hb C (6 Glu→Lys), Hb S (6 Glu→Val), Hb J-Baltimore (16 Gly→Asp) and Hb N-Baltimore (95 Lys→Glu)] was anticipated since all possessed single amino acid substitutions at external residues distant from any subunit interface regions. To adjust the pH and ionic strength of the PMB derivative prior to purification of the PMB chains, the treated hemoglobin was passed through a Sephadex G-25 column (40 × 2.5 cm), previously equilibrated with the appropriate starting buffer. The $alpha_{PMB}$ and $beta_{PMB}$ subunits possess markedly different overall surface charge and, therefore, can be readily purified using selective ion-exchange chromatography.

For the preparation of $alpha_{PMB}$ chains, the Hb AA-PMB derivative solution was charged onto a DE-52 cellulose column (20 × 2.5 cm), equilibrated with 0.01 *M* potassium phosphate buffer, pH 8.15. Under these chromatographic

conditions, the $alpha_{PMB}$ chains were readily eluted, whereas the $beta^{A}_{PMB}$ and unsplit Hb A were retained on the column and discarded. To isolate $beta^{A}_{PMB}$ and several variant $beta_{PMB}$ chains, CM-52 cellulose resin was always employed. For the preparation of $beta^{A}_{PMB}$ chains, the solution of Hb AA-PMB derivative (at the required pH and ionic strength) was placed on a CM-52 cellulose column (10 × 2.5 cm), previously equilibrated with 0.01 *M* potassium phosphate buffer, pH 6.5. The intact Hb A and the $alpha_{PMB}$ chains were retained at this pH and only the $beta^{A}_{PMB}$ chains were eluted. This two column method [7] yielded quantitatively less total hemoglobin chain, but allowed purification of $alpha_{PMB}$ and $beta^{A}_{PMB}$ chains which, after sulfhydryl regeneration, totally recombined to form native Hb A.

We have also employed this CM-52 cellulose cation exchange resin to purify the following variant non-alpha-subunits: $beta^{C}_{PMB}$, $beta^{S}_{PMB}$, $beta^{J\text{-}Baltimore}_{PMB}$ and $beta^{N\text{-}Baltimore}_{PMB}$ (see Fig. 1). The $beta^{C}_{PMB}$ chains, the most positively charged of the beta variant subunits purified, required a resin pH of 7.0 to allow rapid elution of the chains. The Hb CC-PMB derivative was charged onto a CM-52 cellulose column (15 × 2.5 cm), previously equilibrated with 0.01 *M* potassium phosphate buffer, pH 7.0 (see Fig. 1, top). The $beta^{C}_{PMB}$ chains were eluted with starting buffer. These chains were clearly separated from $alpha_{PMB}$ chains whose elution required a salt gradient. Although the overall elution profile is shown, the $alpha_{PMB}$ chains used in our studies were always purified on DE-52 cellulose rather than CM-52 cellulose. The positively charged $beta^{S}_{PMB}$ chains were purified at pH 6.5 on CM-52 cellulose. To prepare these chains from an Hb SS-PMB derivative, a chain procedure identical to that for $beta^{A}_{PMB}$ chains was used (see Fig. 1, middle). The more negatively charged $beta^{J\text{-}Baltimore}_{PMB}$ and $beta^{N\text{-}Baltimore}_{PMB}$ chain purifications required a resin pH of 6.0. To isolate $beta^{J\text{-}Baltimore}_{PMB}$ and $beta^{N\text{-}Baltimore}_{PMB}$ from Hb AJ-Baltimore and Hb AN-Baltimore-PMB derivatives, respectively, an identical procedure was employed. The respective PMB-derivative was charged onto a CM-52 cellulose column (10 × 2.5 cm), previously equilibrated with 0.01 *M* potassium phosphate buffer, pH 6.0 (see Fig. 1, bottom). At this pH the more negatively charged $beta^{N\text{-}Baltimore}_{PMB}$ chains could be readily eluted while the $beta^{A}_{PMB}$ chains were retained. All purified $beta_{PMB}$ chains were immediately titrated to pH 8.15 with sodium hydroxide after elution from the column. This titration to alkaline pH was particularly critical to the stability of $beta^{J\text{-}Baltimore}_{PMB}$ and $beta^{N\text{-}Baltimore}_{PMB}$ chains.

The sulfhydryl groups of the $alpha_{PMB}$ chains were regenerated in the following manner. The solution was brought to a final concentration of 15 m*M* 2-mercaptoethanol and then titrated with hydrochloric acid to a pH of 6.5. The chains were placed on a CM-52 cellulose column (4 × 2.5 cm), previously equilibrated with 0.01 *M* potassium phosphate buffer, pH 6.5 and washed with the same buffer containing 15 m*M* 2-mercaptoethanol for 30 min. The 2-mercaptoethanol was then removed by washing with original buffer for another 30 min and the alpha chains were eluted from the column with 0.02 *M* Tris—HCl buffer, pH 8.0.

The sulfhydryl groups of $beta^{A}_{PMB}$ and the variant $beta_{PMB}$ chains were regenerated as follows. For $beta^{A}_{PMB}$, the solution was titrated to pH 8.15 with sodium hydroxide and charged onto a DEAE-Sephadex A-50 column (6 × 2.5

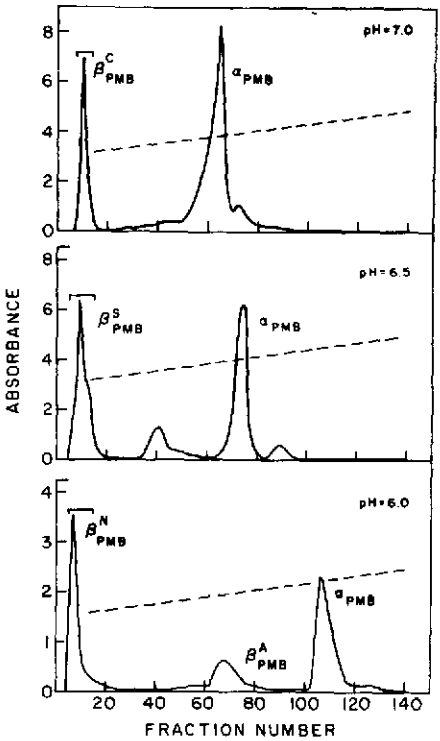

Fig. 1. Purification of variant beta chains on CM-52 cellulose. Top: $Beta^{C}_{PMB}$ chains. A 20-ml vol. (1.6 m*M* in heme) of Hb CC-PMB derivative was loaded onto a 15 × 2.5 cm column previously equilibrated with 0.01 *M* potassium phosphate buffer, pH 7.0. After passage of one column vol. of starting buffer the column was developed with a linear (1000 ml total) gradient of 0 to 0.2 *M* sodium chloride in the same buffer. The flow-rate was 52 ml/h and 5.6-ml fractions were collected. Middle: $Beta^{S}_{PMB}$ chains. A 20-ml vol. (1.8 m*M* in heme) of Hb SS-PMB derivative was charged onto a 10 × 2.5 cm column previously equilibrated with 0.01 *M* potassium phosphate buffer, pH 6.5. Column development was identical to that in the top panel. The flow-rate was 47 ml/h and the fraction size was 5.4 ml. Bottom: $Beta^{N\text{-}Baltimore}_{PMB}$ chains. A 19-ml vol. (1.6 m*M* in heme) of Hb AN-PMB derivative was placed on a 10 × 2.5 cm column previously equilibrated with 0.01 *M* potassium phosphate buffer, pH 6.0. The column was developed as above. A fraction size of 5.8 ml and a flow-rate of 57 ml/h was employed. The elution profile for $beta^{J\text{-}Baltimore}_{PMB}$ purification is quantitatively similar to that of $beta^{N\text{-}Baltimore}_{PMB}$ (not shown). All columns were monitored at 540 nm where the millimolar extinction coefficient is 14.27. In general, overall protein recovery was better at higher pH where the isolated subunits are more stable.

cm), previously equilibrated with 0.01 *M* potassium phosphate buffer, pH 8.15. The chains were regenerated by washing the column for 4 h with a 20 m*M* 2-mercaptoethanol solution made in the same buffer. Since the addition of 2-mercaptoethanol resulted in a decrease in pH, this buffer was always readjusted to pH 8.15 with sodium hydroxide. The 2-mercaptoethanol was then removed by washing with the original buffer for 2 h and the chains were eluted from the column with the stripping buffer, 0.1 *M* potassium phosphate buffer, pH 7.0. Critical adjustments in the $beta^{A}_{PMB}$ regeneration step were necessary to fully regenerate the variant beta chain subunits. The more negatively charged $beta^{J\text{-}Baltimore}_{PMB}$ and $beta^{N\text{-}Baltimore}_{PMB}$ chains bound very tightly to the DEAE-Sephadex A-50 resin and, as such, a reduction in the column

size to 4 × 2.5 cm, as well as addition of 0.5 *M* potassium chloride to the stripping buffer, was necessary. Conversely, the more positively charged $beta^{S}_{PMB}$ chains required an increase in this anion-exchange column size to 12 × 2.5 cm. For the $beta^{C}_{PMB}$ chains, a QAE-Sephadex A-50 column (6 × 2.5 cm) was generally employed for the regeneration step.

The PMB spectrophotometric titration method [8] can be used to quantitate the number of free sulfhydryl groups (i.e., to determine if regeneration is complete) as well as evaluate purity of the isolated subunit. The theoretical number of available cysteine groups is 1.0 for alpha chains (alpha 104) and 2.0 for beta chains (beta 93 and beta 112). The experimental number of sulfhydryls titrated were 1.05 ± 0.08 for alpha chains, 1.94 ± 0.14 for $beta^{A}$ chains and 1.92 ± 0.14 for the variant beta chains. These findings not only reveal complete regeneration of the heme subunits (> 95%) but also provide an independent criteria for sample homogeneity.

Zone electrophoresis of the four variant beta chain subunits, as well as normal beta chains, was carried out on cellulose acetate and the results are depicted in Fig. 2. Apparent homogeneity exists for all these hemoglobin subunits. Furthermore, all beta subunits when mixed with their complementary alpha chains formed reconstituted hemoglobins electrophoretically identical to the unsplit parent hemoglobin tetramers. Complete recombination of a subunit with its dissimilar partner is an essential test of nativeness. Functional studies also support our electrophoretic results on hemoglobin reconstitution. Isolated hemoglobin subunits exhibit high ligand affinity and bind oxygen noncooperatively. Appearance of the familiar sigmoidal oxygen saturation curve only occurs upon reconstitution of a fully functioning hemoglobin tetramer from native subunits. Results of functional studies on three represen-

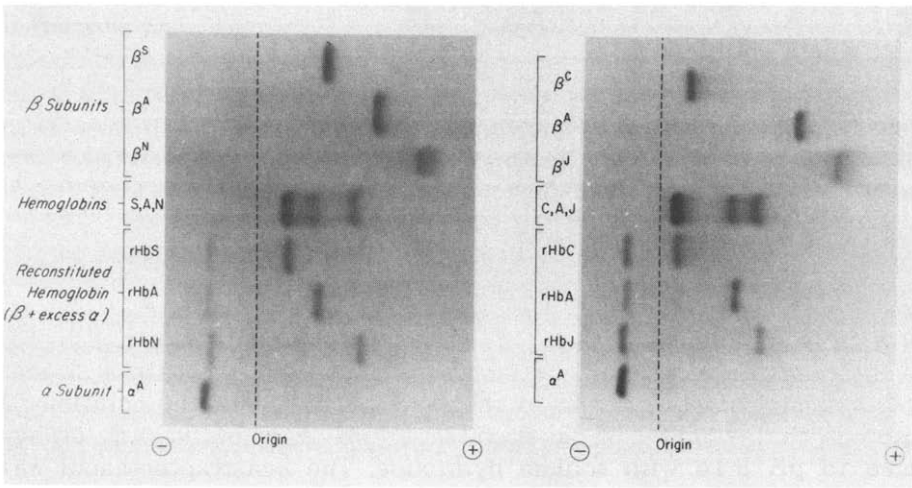

Fig. 2. Electrophoresis of the isolated beta heme subunits and reconstituted parent hemoglobins of S, C, A, N-Baltimore and J-Baltimore. Mixtures containing unsplit normal and variant hemoglobins are also included as controls. The beta chains were over titrated with their complementary alpha chains to demonstrate complete recombination. The electrophoresis was performed on cellulose acetate in Tris—borate—EDTA buffer, pH 8.6 and stained with Ponceau S.

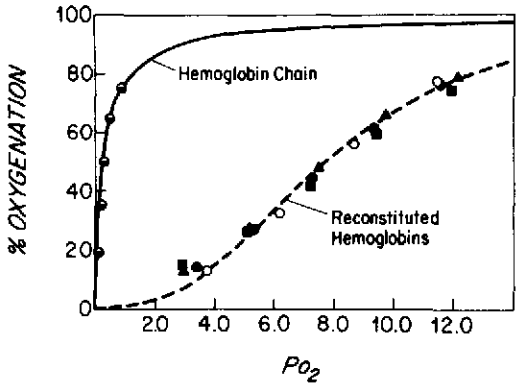

Fig. 3. Oxygen saturation curves of r Hb N-Baltimore (▪—▪), r Hb S (▲—▲) and r Hb A (●—●) in 0.1 *M* potassium phosphate buffer, pH 7.0 and 20°C. The saturation curves of a normal Hb A hemolysate (○—○) and isolated alpha chains are included as controls. The protein concentration was 50 μmol/l in heme and these experiments were performed on a 50:50 mixture of alpha and beta chains (heme basis). The P_{50} (and *n* value) were 8.0 mm Hg (2.5), r Hb N-Baltimore; 7.7 mm Hg (2.8), r Hb A; 7.6 mm Hg (2.8), r Hb S; 7.7 mm Hg (2.8), Hb A hemolysate.

tative reconstituted hemoglobins appear in Fig. 3, and demonstrate that r HbN-Baltimore (a reconstituted negatively charged variant), r Hb A and r Hb S (a positively charged reconstituted hemoglobin) exhibit oxygen affinity and cooperativity indicative of normal hemoglobin tetramers.

The experimental protocols described above gave the following yields as percentage of total hemolysates: 18—22% for alpha chains, 10—14% for beta chains from homozygotes, 4—8% for beta chains from heterozygotes. The PMB derivative and column procedures described here could be readily scaled up ten-fold and the larger preparations normally yielded greater amounts of chains (2—4% increase). These purification techniques can be readily extrapolated to the isolation of a variety of non-alpha-subunits with widely distinct electrostatic properties. These subunits are fully native and can be readily employed to study a wide variety of biochemical, immunological, functional and subunit assembly events of hemoglobin.

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