Demonstration of the Hybrid Hemoglobin $\alpha^2\beta^A\beta^S$ *

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SUMMARY

By using the cross-linking reagent $p,p'$-difluoro-$m,m'$-dinitrodiphenylsulfone, we fixed in the tetramer form the various species of hemoglobin present in a mixture of hemoglobin A and hemoglobin S and demonstrated that the species $\alpha^A\beta^A\beta^S$ exists.

The erythrocytes of individuals carrying the genetic trait for sickle cell anemia are known to contain both normal hemoglobin A and hemoglobin S (2). It is widely accepted that under physiological conditions hemoglobin exists as an equilibrium mixture of tetramer ($\alpha 2 \beta 2$) and dimer subunits ($\alpha \beta$) (3).

$$\alpha 2 \beta 2 \rightleftharpoons \alpha \beta \alpha \beta$$

In erythrocytes containing both hemoglobin A and hemoglobin S, it has been predicted (4) that a third tetramer species, an asymmetric hybrid of hemoglobin A and hemoglobin S, should exist.

$$\alpha^A\beta^A\beta^S \rightleftharpoons \alpha^A\beta^A + \alpha^A\beta^S$$

If it is assumed that $K_1 = K_2 = K_3$, and that the initial concentrations of $\alpha^A\beta^A\beta^S$ and $\alpha^A\beta^A\beta^S$ are equal, then at equilibrium it can be shown that the concentrations of $\alpha^2\beta^A\beta^A$, $\alpha^A\beta^A\beta^S$, and $\alpha^A\beta^A\beta^S$ are equal.

The $\beta^S$ polypeptide chain differs from the $\beta^A$ polypeptide chain only at the sixth position from the NH$_2$-terminal end, where a valyl residue replaces the normal glutamyl residue (5). As a result of this substitution, hemoglobin S has two fewer negative charges at neutral pH than hemoglobin A; therefore, it has a lower mobility toward the anode during electrophoresis and is eluted behind hemoglobin A when the two are chromatographed together on cation exchange resins. Accordingly, it is expected that $\alpha^A\beta^A\beta^S$ would have only one less negative charge than hemoglobin A at physiological pH since it has only one $\beta^S$ polypeptide chain. Thus, $\alpha^A\beta^A\beta^S$ should move in a position between hemoglobin A and hemoglobin S during separation of a mixture of these hemoglobins by electrophoresis or chromatography on cation exchange resins.

Heretofore, $\alpha^A\beta^A\beta^S$ has never been isolated, presumably because the tetramer-dimer equilibrium is very rapid compared to the separation technique employed (3). That is, during separations of mixtures of hemoglobin A and hemoglobin S, $\alpha^A\beta^A\beta^S$ molecules are not detected because they dissociate into $\alpha^2\beta^A$ and $\alpha^A\beta^S$ dimers and these recombine with like dimers to reform hemoglobin A and hemoglobin S. Hence, if the hybrid tetramer is to be detected, it is necessary to fix it in the tetramer form.

The reaction of hemoglobin with FNPS forms an intramolecular cross-link between the $\alpha$ subunits of hemoglobin at the NH$_2$-terminals of the $\alpha$ chains (6), providing a method for fixing hemoglobin molecules in the tetramer form. This paper describes the use of this cross-linking reaction to demonstrate the presence of $\alpha^A\beta^A\beta^S$ in a mixture of CO hemoglobin A and CO hemoglobin S.

EXPERIMENTAL PROCEDURE

**Materials**—FNPS was obtained from Pierce Chemicals and was purified as previously described (8). Hemolysates were prepared as before (7) from out-of-date blood bank blood provided by the City of Memphis Hospitals. Human CO hemoglobin A and human CO hemoglobin S were isolated from hemolysates of blood from donors carrying the genetic trait for sickle cell anemia by a modification of the chromatographic procedure described previously (8). In the modified isolation, the hemolysate was dialyzed against three changes of pH 0.40 phosphate buffer (6.211 g of NaH$_2$PO$_4$·H$_2$O and 2.598 g of Na$_2$HPO$_4$ per liter) and then pumped onto the column which had been previously equilibrated with pH 6.46 phosphate buffer (7.175 g of NaH$_2$PO$_4$·H$_2$O and 3.262 g of Na$_2$HPO$_4$ per liter). A linear phosphate buffer gradient was used to develop the column. The mixing vessel for the gradient initially contained pH 0.40 phosphate buffer and the second vessel contained an equal volume of pH 9.20 phosphate buffer (5.725 g of Na$_2$HPO$_4$ per liter) and then pumped onto the column which had been previously equilibrated with pH 6.46 phosphate buffer (7.175 g of NaH$_2$PO$_4$·H$_2$O and 3.262 g of Na$_2$HPO$_4$ per liter). The result of a typical isolation is shown in Fig. 1.

**Hemoglobin Concentration**—CO hemoglobin was converted to cyanmethemoglobin (9), and the concentration was determined spectrophotometrically with $\varepsilon_{540} = 11000$ M$^{-1}$ cm$^{-1}$ per heme (10).

**Reaction and Isolation of Reacted Hemoglobins**—The reaction of human CO hemoglobin with FNPS, the isolation of the cross-linked hemoglobin tetramers by gel filtration, and the fractionation of the cross-linked tetramers by ion exchange chromatography on a sulfoethyl Sephadex column were carried out as

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previously reported (6) with minor modifications. The reaction procedure was modified in that the 1% CO hemoglobin solution was 0.5% in CO hemoglobin A and 0.5% in CO hemoglobin S. Ion exchange chromatography was done on a jacketed (4") column (5 x 60 cm) of sulfoethyl Sephadex which was developed with a phosphate buffer gradient. The two buffers used to make the gradient were: 1.6 liters of pH 6.66, μ = 0.1 M phosphate (4.552 g of NaH₂PO₄·HzO and 3.122 g of Na₂HPO₄ per liter) and 1.6 liters of pH 9.2, μ = 0.1 M phosphate (5.725 g of Na₂HPO₄ per liter). Sulfoethyl Sephadex was used instead of sulfoethyl Sephadex in some experiments and gave identical results.

Starch Gel Electrophoresis—Vertical starch gel electrophoresis was performed by the method of Smithies (11) with a buffer described by Boyer et al. (12).

Ion Exchange Chromatography of Tryptic Peptides—Heme was removed from hemoglobin samples as before (6). Tryptic hydrolysis of the globin was carried out by a procedure used for the β chain of hemoglobin (13). The method used to analyze the tryptic peptides was a modified version of a published procedure (14). Aminex 4, a bead-form sulfonic acid resin (Bio-Rad Laboratories) was used in the short column (0.9 x 16 cm) of a Spinco amino acid analyzer (model 120A). The pyridine-acetate buffers were prepared according to the established procedure (14) but were diluted with water to two-thirds of their original concentration before using. Dimethylsulfoxide ninhydrin solution (15) was used. The buffer gradient was pumped at a rate of 60 ml per hour, and the ninhydrin solution was pumped at a rate of 30 ml per hour.

RESULTS

After cross-linking with FNPS, the reaction mixture was dialyzed and concentrated as before (6). A sample of the reaction mixture was analyzed by starch gel electrophoresis and compared with control samples. The results (Fig. 2) show that cross-linking of hemoglobin A and hemoglobin S does yield a third hemoglobin species which moves in a position between hemoglobin A and hemoglobin S, where α₄β₄ hemoglobin was expected to move. Thus, it appears that cross-linking with FNPS has caught α₄β₄ and α₄β₄ dimers in the act of association, as predicted.

In order to characterize the α₄β₄β₄ hemoglobin, it was isolated from the reaction mixture and purified. The cross-linked hemoglobin was separated from hemoglobin free to dissociate into dimers by passing the reaction mixture through a Sephadex G-100 column in the presence of 1 M MgCl₂. In 1 M MgCl₂, hemoglobin is normally completely dissociated into dimer subunits (16) so that the hemoglobin which has been fixed in the tetramer form by cross-linking with FNPS will emerge from the column before the unreacted hemoglobin dimers. The pooled fractions (indicated on Fig. 3) from this separation were...
dialyzed free of MgCl₂, concentrated to approximately 5% by ultrafiltration, and chromatographed on a column of sulfopropyl Sephadex. The result of this treatment is shown in the upper elution pattern of Fig. 4. The lower elution pattern of Fig. 4 shows a chromatogram under identical conditions of a mixture of cross-linked hemoglobin A and cross-linked hemoglobin S. Prior to the chromatography shown in the lower elution pattern of Fig. 4, hemoglobins A and S were reacted separately, the cross-linked species were isolated separately by gel filtration on the Sephadex G-100 column in the presence of 1 M MgCl₂, and then the cross-linked hemoglobins A and S were mixed together. The direct comparison of the hemoglobins A and S, mixed and then reacted with FNPS (Fig. 4, upper), to hemoglobins A and S, mixed after reaction, shows that only in the former experiment does the predicted third cross-linked tetramer species (HbAS in Fig. 4, upper) appear. Previous studies (6) have shown that hemoglobin molecules which were reacted and purified by procedures used for the samples in Fig. 4 are tetramers with an intramolecular cross-link between the NH₂-terminal valines of the α chains. The remote possibility that the HbAS peak (Fig. 4, upper) represents an aggregate of cross-linked hemoglobin A and cross-linked hemoglobin S tetramers can be ruled out since no species larger than tetramers are observed during the separation on Sephadex G 100 column in the presence of 1 M MgCl₂. Furthermore, the lower elution pattern of Fig. 4 shows no middle peak and thus no aggregation occurs when the cross-linked tetramers of hemoglobin A and hemoglobin S are mixed after 1 M MgCl₂ has been removed.

To prove that the pooled fractions of HbAS (Fig. 4, upper) are cross-linked α₂β₂β₂, it is necessary to show that the HbAS tetramers contain both β A and β S chains. By using the chromatographic procedure which has been used to differentiate hemoglobin A from hemoglobin S (14), tryptic peptides of the HbAS from the fractions indicated on Fig. 4 (upper) were compared to tryptic peptides from hemoglobins A and S. The results (Fig. 5) show that HbAS contains equal amounts of the β A and β S NH₂-terminal tryptophan peptides. This confirms that HbAS of Fig. 4 (upper) is cross-linked α₂β₂β₂.

**DISCUSSION**

Under the conditions used in this study, the reaction of FNPS with CO hemoglobin is highly specific for the formation of an intratetrameric cross-link between the NH₂-terminal valines of the α chains (6). With the assumption that K₁ = K₂ = K₃, if a cross-linking reaction is carried out on a mixture of equal amounts of hemoglobin A and hemoglobin S, it is expected that equal amounts of cross-linked α₂β₂, cross-linked α₂β₂, and cross-linked α₂β₂ will be formed. This is approximately what is found, indicating that the cross-linking occurs in the same manner as α₂β₂ and α₂β₂. It has been assumed that the cross-linking occurs within the tetramers because in the tetramer form the amino groups of the ends of the α chains are the proper distance apart and in the right configuration on the molecule's surface to be cross-linked (6). Probably, once one
fluorine of FNPS has been displaced within a tetramer, the effective concentration of FNPS in the vicinity of the other NH2-terminal valine is very high and the cross-linking is quickly completed. Although the tetramer dimer equilibrium is greatly in favor of the tetramer, FNPS could react at one end with the NH-terminal valine of the α chain of an αα dimer and then wait upon reassociation with another αβ dimer before cross-linking is completed. Also, FNPS could react at one end with a tetramer, which could dissociate and then reassociate with another dimer, and then FNPS could react at the other end to complete cross-linking and form an artificial hybrid. Either of these mechanisms of cross-linking will necessarily take longer to complete than cross-linking within an undissociated tetramer because the same conformation is required for cross-linking to occur and reassociation of dimers requires time regardless of how rapidly it happens. Thus, since there is a very large population of tetramers in the proper conformation for cross-linking compared to a relatively small amount of dimers, which could only be cross-linked by a more complex mechanism, it seems most likely that cross linking is accomplished within individual tetramers.

The possibility that the cross-linking reaction creates an artificial molecule other than fixing a naturally occurring hybrid tetramer is considered to be very unlikely. Since cross-linked ααββ is formed to approximately the same extent as cross-linked ααβA and cross-linked ααβB, it is probably cross-linked by the same mechanism and, for the reasons given above, this mechanism is most likely an intratetrameric cross-link.

The amount of cross-linked ααβA (241 mg) in the lower graph of Fig. 4 is slightly less than the amount of cross-linked ααβA (257 mg). This difference is exaggerated by the pH gradient of the eluting buffer which spreads out the cross-linked ααβA fractions over a larger volume than the cross-linked ααβA fractions. Although this same spreading effect is acting in the upper graph of Fig. 4, it appears that the amount of cross-linked ααβA is greater than the amount of cross-linked ααβA and cross-linked ααβA. This probably means that, contrary to the original assumption, K2 < K1 and K3 and thus ααβA not only exists in mixtures of ααβA and ααβA, it is the predominant species present.

Since the pioneering work of Itano (17), there has been considerable interest in detecting and isolating hybrid hemoglobin tetramers. In most cases the detection and isolating methods have depended on the hybrid molecule's having a charge different from both its precursor hemoglobins. Some of the first hybrids detected were "unnatural" in the sense that part of the charge difference was produced by oxidized heme iron in the α or β chains (18). Other "unnatural" hybrids have been produced by asymmetric recombination of hemoglobins of unlike species under dissociating conditions, e.g. recombination of canine and human hemoglobins (19–21). Guidotti (23) has shown by means of osmotic pressure measurements that hybrid tetramers are present in mixtures of horse and human hemoglobins, in mixtures of normal and chemically modified hemoglobins, and in mixtures of liganded hemoglobin and deoxyhemoglobin. More recently (23) a naturally occurring hybrid of human hemoglobin has been reported and studied. All of these studies provide strong circumstantial evidence for the existence of the hybrid hemoglobin, ααβAβB. This hybrid is of particular interest because a recent explanation for the relatively greater resistance to sickling of erythrocytes from individuals carrying the trait for sickle cell anemia, compared to erythrocytes from individuals homozygous for hemoglobin S, is based on the assumption that ααβAβB hemoglobin molecules are present in the linear aggregates formed during sickling and that they tend to limit the aggregation (24). Now that the postulated hybrid molecule has been demonstrated, hypotheses such as the one just cited, which assume the presence of the hybrid, are better founded.

The method described in this paper should be useful for demonstrating other hybrid hemoglobins where the hybrid in question bears a charge different from the hemoglobins from which it was formed.

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