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Cross-Linking Reaction of a Valency Asymmetrical Hybrid Hemoglobin Formed in a Mixture of Hemoglobin A and Methemoglobin

KIYOMI KIKUGAWA* and MASUMI ARAI

Tokyo College of Pharmacy, 1432–1 Horinouchi, Hachioji, Tokyo 192–03, Japan

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It is known that hemoglobin A [Hb A ($\alpha_2\beta_2$)] is cross-linked by reaction with double-headed aspirin [bis (3,5-dibromosalicyl)fumarate], and the cross-linking site is the β-cleft of the tetramer, the binding site of 2,3-diphosphoglycerate. Reaction of methemoglobin [MetHb ($\alpha_2^+\beta_2^+$)] with the reagent resulted in the formation of MetHbFu, which was confirmed to be a MetHb tetramer intramolecularly cross-linked at the same site. Unstable valency asymmetrical hybrid hemoglobin [Hb AM ($\alpha\alpha^+\beta\beta^+$)], which is assumed to be present in a mixture of Hb A and MetHb, could be fixed as a stable tetramer [HbAMFu] by the cross-linking reaction. The intramolecular cross-links in HbAMFu were at the same β-cleft. It is suggested that MetHb forms the valency asymmetrical hybrid in the presence of Hb A.

Keywords——hemoglobin A; methemoglobin; valency asymmetrical hybrid hemoglobin; bis(3,5-dibromosalicyl)fumarate; cross-linking reaction

Hemoglobin A (Hb A) is an allosteric protein molecule composed of 2α-subunits and 2β-subunits, each bearing a heme moiety in the ferrous state ($\alpha_2\beta_2$). The heme moiety in the ferrous state can bind and release oxygen, and transfers oxygen from the lungs to tissues.[1] Many studies have been done on the formation of methemoglobin (MetHb), in which all the subunits are in the ferric state ($\alpha_2^+\beta_2^+$),[2] but it is unlikely that all the hemoglobins are completely oxidized into the ferric state *in vivo*. It is important to investigate where the heme moiety in the ferric state is located in partially oxidized hemoglobins. In earlier studies,[3–6] the presence of heterogeneous tetramers composed of both ferrous and ferric forms was suggested. The presence of symmetrical hybrids, ($\alpha_2^+\beta_2$) and ($\alpha_2\beta_2^+$), has been demonstrated by the use of the isoelectrofocusing technique.[7,8] Perutz[9] suggested that valency asymmetrical hybrid molecules [Hb AM ($\alpha\alpha^+\beta\beta^+$)] are unstable and readily split into the unlike dimers, which recombine to form stable symmetrical hybrids under analytical conditions. Perrella *et al.*[10] demonstrated the presence of valency asymmetrical hybrids in a mixture of Hb A and MetHb by electrophoresis at subzero temperature.

It has been shown that a cross-linking agent, double-headed aspirin [bis(3,5-dibromosalicyl)fumarate] (Chart 1),[11,12] can form intramolecular cross-links between $\beta_1$82 Lys and $\beta_2$82 Lys of Hb A and Hb S tetramers, the binding site of 2,3-diphosphoglycerate (DPG).[12,13] This reagent produces intramolecular cross-links in the unstable asymmetrical

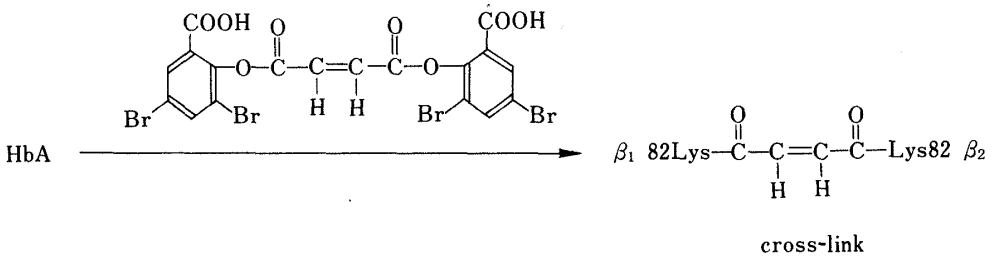

Chart 1

hybrids between Hb A and abnormal hemoglobins.[14] Miura and Ho[15] prepared cross-linked valency asymmetrical hybrids of Hb A and Hb C for their nuclear magnetic resonance (NMR) studies. However, the profiles of the cross-linking reaction of the unstable asymmetrical valency hybrids by the reagent have not yet been well characterized. This communication deals with the formation of intramolecular cross-links in MetHb and in the unstable valency asymmetrical hybrid [Hb AM ($\alpha\alpha^+\beta\beta^+$)] present in a mixture of Hb A and MetHb.

Experimental

Absorption spectra were measured with a Shimadzu model UV 200S double-beam spectrophotometer. Isoelectrofocusing was performed on an Ampholine PAG plate (pH 5.5—8.5) (LKB Aminkemi) after prerunning for 1 h (LKB 2117 Multiphor). The plate was stained with bromochlorophenol blue. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done on a 10% slab gel with the LKB 2117 Multiphor after prerunning for 30 min. The slab was stained with Coomassie Brilliant Blue R-250. A linear relationship between the mobilities and the log molecular weights was obtained with the molecular weight standards, bovine serum albumin (67000 daltons), ovalbumin (43000 daltons), chymotrypsinogen A (25000 daltons) and ribonuclease A (13700 daltons). Gel filtration was performed on a column of Ultrogel AcA 44 (2 × 62 cm) (LKB Aminkemi) equilibrated with 0.025 M Tris–HCl (pH 7.0) containing 1 M $MgCl_2$. The void volume of the column was 70 ml (estimated by using blue dextran 2000). Hemoglobins were purified by chromatography on CM Sephadex C-50 (Pharmacia Fine Chemicals) or by chromatofocusing with PBE 94 and polybuffer 96 (pH 6.8—7.0) (Pharmacia Fine Chemicals).

Hemoglobin solution was concentrated through a Diaflow membrane PM 10 or PM 30 (Amicon Corporation) under nitrogen gas. The CO-form of hemoglobin was converted into the oxy-form by photolysis using a rotary evaporator.[16] Hemoglobin concentration was determined spectrophotometrically by using millimolar extinction coefficients of $m\varepsilon_{569}=13.4$[17] for the CO-form, $m\varepsilon_{577}=14.6$ for the oxy-form[17] and $m\varepsilon_{630}=4.01$[18] for the met-form, on a heme basis. Isosbestic points of the CO-form and met-form were found at 518 and 587 nm ($m\varepsilon_{518}=7.15$). The content of met-subunit in hemoglobin tetramers was calculated from the absorbance at 630 nm (absorption maximum of met-form) and that at 518 nm (isosbestic point of CO-form and met-form). Oxygen equilibrium curves of oxyhemoglobin were determined in 0.1 M phosphate (pH 7.0) in the presence and absence of inositol hexaphosphate (IHP) (Sigma Chemical Company) at 20 °C.

Bis(3,5-dibromosalicyl)fumarate——The reagent was prepared according to the method of Walder *et al.*[11] Recrystallization from ethyl alcohol gave white needles melting at 195—199 °C (lit. 195—196 °C[11]). NMR (d_6-dimethylsulfoxide) ppm: 8.36 (2H, d, $J=2$ Hz), 8.12 (2H, d, $J=2$ Hz), 7.36 (2H, s), 3.48 (2H, q, $J=7$ Hz), 1.08 (3H, t, $J=7$ Hz) indicated that it contained 1 mol of ethyl alcohol. *Anal.* Calcd for $C_{18}H_8Br_4O_8\cdot C_2H_6O$: C, 33.36; H, 2.24. Found: C, 33.08; H, 2.16.

Hb A and MetHb——The packed red cells from normal human blood were washed with saline and hemolyzed in 5 volumes of 5 mM ethylenediaminetetraacetic acid–5 mM phosphate (pH 7.5). The suspension was centrifuged at 13000 rpm for 10 min after addition of 1/10 volume of 9% NaCl to remove ghosts. The supernatant hemolysate was bubbled with CO gas, and the hemolysate (corresponding to 0.5 g of Hb A) was applied to a column of CM Sephadex C-50 (2 × 43 cm) equilibrated with 0.01 M phosphate (pH 7.0). Hb A was eluted in a linear gradient fashion from pH 7.0 to 8.0 with 0.01 M phosphate. CO-liganded Hb A was eluted between pH 7.3 and 7.5. Unless otherwise mentioned, Hb A was used in the CO-liganded form, because CO protects hemoglobin from oxidation and because the conformation of CO Hb A is very similar to that of oxy Hb A.[19]

The CO Hb A was treated with 40-fold excess of potassium ferricyanide in the phosphate buffer at room temperature for 4 h. After removal of ferricyanide by passing a solution containing 0.5 g of MetHb through a column of Sephadex G-25, the MetHb was purified through a column of CM Sephadex C-50 (0.8 × 20 cm) as described above. MetHb was eluted between pH 7.65 and 7.75.

Hb A Cross-Linked with Bis(3,5-dibromosalicyl)fumarate (HbFu)——CO-liganded HbFu was prepared as described.[12] It was purified on a CM Sephadex C-50 column with a linear pH gradient from pH 6.8 to 8.0. The modified hemoglobin was eluted between pH 7.1 and 7.3.

MetHb Cross-Linked with Bis(3,5-dibromosalicyl)fumarate (MetHbFu)——A mixture of MetHb (2.8 mM on a heme basis) and 0.71 mM bis(3,5-dibromosalicyl)fumarate in 0.01 M phosphate (pH 7.5) was incubated at 37 °C for 2 h. The reaction mixture was passed through a column of Sephadex G-25 equilibrated with 0.01 M phosphate (pH 7.0). The fraction containing hemoglobin was applied to a column of CM Sephadex C-50 (1.6 × 25 cm) equilibrated with the phosphate buffer (pH 7.0). The hemoglobins were eluted with a linear pH gradient from pH 7.0 to 8.3 with 0.01 M phosphate. The result is shown in Fig. 1.

Hb AM Cross-Linked with Bis(3,5-dibromosalicyl)fumarate (HbAMFu)——To a mixture of CO-liganded Hb A (1.68 mM on a heme basis) and MetHb (1.64 mM on a heme basis) in 0.01 M phosphate (pH 7.4) was added an equal volume of a solution of 0.83 mM bis(3,5-dibromosalicyl)fumarate. The mixture was incubated at 37 °C for 2 h. The

reaction mixture was passed through a column of Sephadex G-25 equilibrated with 0.025 M Tris–HCl (pH 8.0), and applied to a column of PBE (1.9 × 34 cm) equilibrated with the same buffer. The reaction products were eluted with 20-fold diluted polybuffer 96 (pH 7.0). The result is shown in Fig. 8.

Oxidation of HbAMFu by Nitrite——CO-liganded HbAMFu (1.6 mM on a heme basis) was treated with 7.7 mM $NaNO_2$ in 0.1 M phosphate (pH 7.0) at room temperature for 3 h. The reaction mixture was passed through a column of Sephadex G-25 to remove the salt.

Reduction of MetHbFu by Ascorbic Acid——An equal volume of MetHbFu (1.6 mM on a heme basis) in 0.1 M phosphate (pH 7.0) and 9.5 mM ascorbic acid in the phosphate buffer were mixed under anaerobic conditions in a Thunberg tube. The mixture was allowed to stand at room temperature for 150 min, then immediately passed through a column of Sephadex G-25 equilibrated with 0.025 M Tris–HCl (pH 8.3). The eluate was bubbled with CO gas and applied to a PBE column (1 × 45 cm) to separate the reduction products. The result is shown in Fig. 5.

Reduction of HbAMFu by Ascorbic Acid——An equal volume of CO-liganded HbAMFu (1.2 mM on a heme basis) in 0.1 M phosphate (pH 7.0) and 6.0 mM ascorbic acid in the phosphate buffer were mixed under anaerobic conditions, and the mixture was allowed to stand at room temperature for 150 min. The reaction mixture was treated as above.

Results

Cross-Linking Reaction of MetHb with the Double-Headed Aspirin

Although the cross-linking reaction of oxy- or CO-liganded hemoglobins by the double-headed aspirin has been well characterized,[11-14] it is not known whether methemoglobins undergo similar modifications. In order to obtain information on the profile of the cross-linking reaction of MetHb, the reaction of MetHb with the double-headed aspirin was performed.

MetHb was reacted with an equimolar amount of the double-headed aspirin at pH 7.5 and 37 °C for 2 h. The reaction mixture was passed through a column of Sephadex G-25, then applied to a column of CM Sephadex C-50. The major reaction product (MetHbFu) was eluted faster than MetHb, and the yield was estimated to be 82% by spectrophotometry (Fig. 1). The purified MetHbFu was found to be homogeneous and was focused at a more acidic

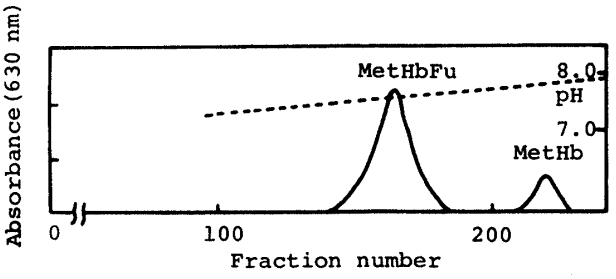

Fig. 1. CM Sephadex C-50 Column Chromatography of the Reaction Mixture of MetHb and Bis(3,5-dibromosalicyl)fumarate

The reaction mixture (4 ml) of 2.8 mM MetHb and 0.71 mM reagent was applied to a column (1.6 × 25 cm), and the hemoglobins were eluted with a pH gradient. Ten milliliter fractions were collected.

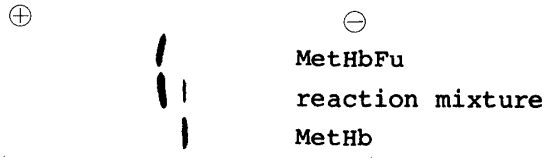

Fig. 2. Isoelectrofocusing Pattern of the Reaction Mixture of MetHb and Bis(3,5-dibromosalicyl)fumarate, of MetHbFu Purified by CM Sephadex C-50 Column (Fig. 1)

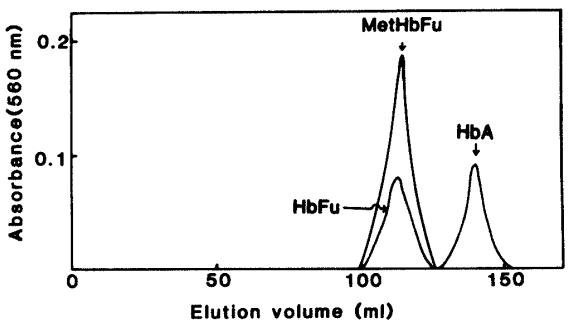

Fig. 3. Gel Filtration Profile of Hb A, HbFu and MetHbFu on an Ultrogel AcA 44 Column (2 × 62 cm) Equilibrated with 0.025 M Tris–HCl (pH 7.0) Containing 1 M $MgCl_2$

position than that of MetHb on isoelectrofocusing (Fig. 2). Formation of MetHbFu was significantly inhibited in the presence of IHP (data not shown), suggesting that the modification took place at the DPG binding site of MetHb tetramer, as is the case in the modification of Hb A.[12] It has been demonstrated that Hb A is dissociated into dimers in the presence of 1 M $MgCl_2$.[20] When MetHbFu was passed through a column of Ultrogel AcA 44 in the presence of 1 M $MgCl_2$ it was eluted much faster than Hb A, and appeared at the same position as HbFu, a cross-linked product of Hb A with the double-headed aspirin (Fig. 3). The result indicates that MetHbFu had a cross-link which did not allow the product to dissociate into dimers even at a high salt concentration.[15]

In order to elucidate the structure of MetHbFu, its conversion into HbFu, whose structure was firmly established by X-ray analysis,[12] was attempted. MetHbFu was reduced with ascorbic acid under anaerobic conditions, and the products were analyzed by isoelectrofocusing. Four bands were observed at more acidic positions than that of MetHbFu (Fig. 4). The standard HbFu was not altered under the same conditions, indicating that the fumaryl group was not affected by the treatment. The four products, R-1, R-2, R-3 and R-4, were separated and purified by chromatofocusing as CO-liganded forms (Fig. 5), and they were each found to be homogeneous on an isoelectrofocusing gel (Fig. 4). Product R-4 showed the same isoelectric point and absorption spectrum as HbFu did. The other products R-1, R-2 and R-3 focused between HbFu and MetHbFu, and showed higher isoelectric points in that order (Fig. 4). Spectrophotometric estimation of R-1, R-2 and R-3 revealed that the ratio of absorbance at 630 nm to that at 569 nm was higher in that order, and analysis of the content of met-subunit in each product indicated that R-1 contained 70.3% met-subunit, R-2, 48.6%; and R-3, 25.7%. The results demonstrated that the products R-1, R-2 and R-3 are the intermediates of the reduction of MetHbFu, bearing 3 met-subunits, 2 met-subunits and one met-subunit, respectively.

The oxygen affinity of R-4 was compared with those of HbFu and Hb A (Fig. 6). The P_{50} value of R-4 (5.0 mmHg) and the Hill's coefficient (2.5) were close to those of HbFu (4.2 mmHg and 2.5, respectively), and the P_{50} value was not shifted in the presence of IHP.

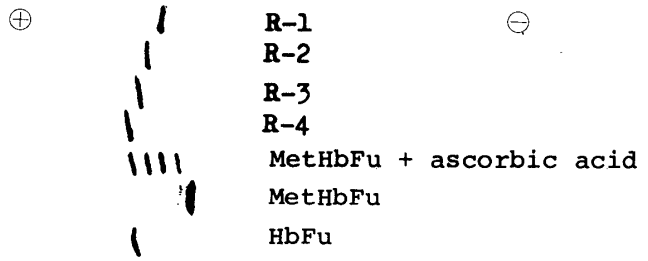

Fig. 4. Isoelectrofocusing Pattern of MetHbFu Reduced by Ascorbic Acid

The products R-1, R-2, R-3 and R-4 were obtained by chromatofocusing (Fig. 5).

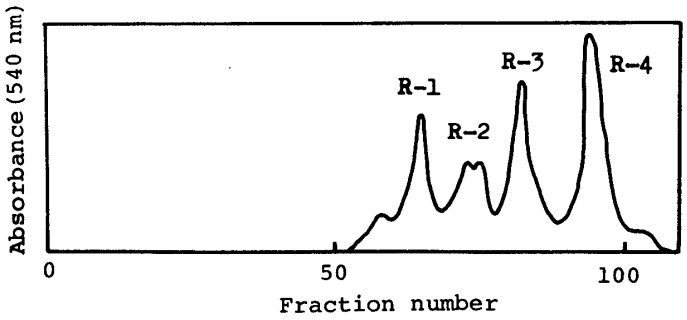

Fig. 5. Chromatofocusing Pattern of MetHbFu Reduced by Ascorbic Acid

The reaction mixture (2 ml) containing 0.80 mM MetHbFu and 4.25 mM ascorbic acid was applied to a PBE column (1 × 45 cm) equilibrated with 0.025 M Tris–HCl (pH 8.3), and the hemoglobins were eluted with polybuffer 96 (pH 7.0) with 17-fold dilution. Two milliliter fractions were collected.

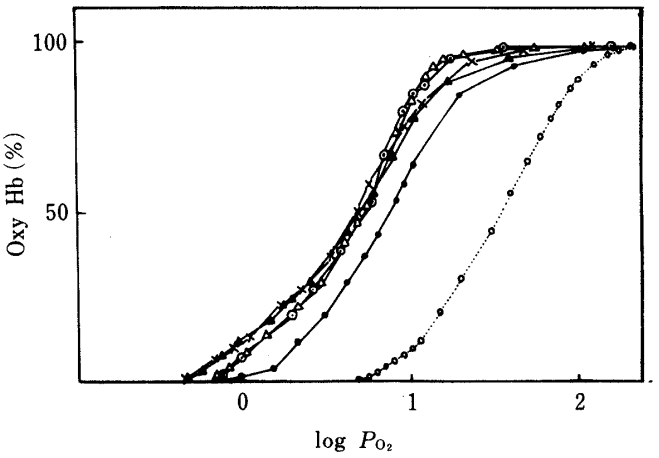

Fig. 6. Oxygen Binding Curves for Hb A (—●—), Hb A+2 mM IHP (---○---), HbFu (—×—), HbFu+2 mM IHP (—▲—), R-4 (—△—), and R-4+2 mM IHP (—⊙—) in 0.1 M Phosphate (pH 7.0) at 20 °C

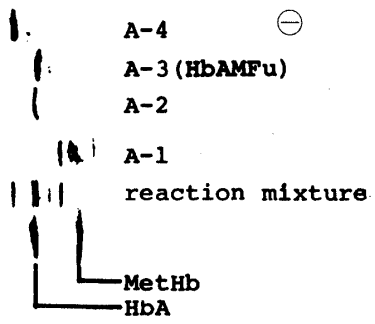

Fig. 7. Isoelectrofocusing Pattern of the Reaction Mixture of Hb A, MetHb and Bis(3,5-dibromosalicyl)fumarate, and the Products A-1, A-2, A-3 and A-4 Purified by Chromatofocusing (Fig. 8)

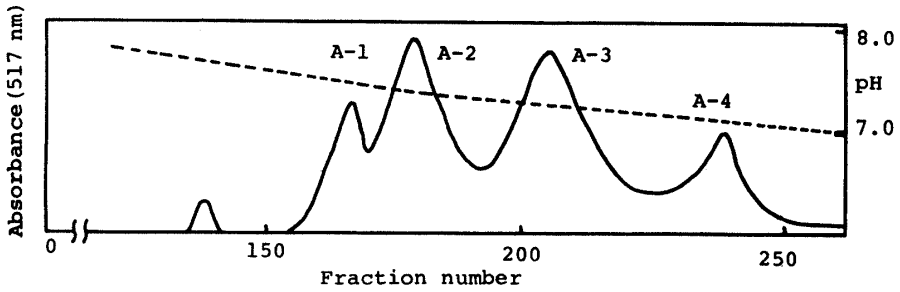

Fig. 8. Chromatofocusing of the Reaction Mixture of Hb A, MetHb and Bis(3,5-dibromosalicyl)fumarate

The reaction mixture (4 ml) containing 0.84 mM Hb A, 0.82 mM MetHb and 0.42 mM reagent was applied to a PBE column (1.9 × 34 cm) equilibrated with 0.025 M Tris–HCl (pH 8.0), and the hemoglobins were eluted with polybuffer 96 (pH 7.0) at 20-fold dilution. Two milliliter fractions were collected.

In contrast, Hb A showed the P_{50} value of 7.4 mmHg, which was shifted to 36 mmHg in the presence of IHP, and the Hill's coefficient was 2.8. The absence of a shift in the P_{50} value of HbFu was presumably due to the cross-linking between β_1 82 Lys and β_2 82 Lys by the fumaryl group, at the 2,3-DPG binding site.[12] The absorption spectrum, the isoelectric point, and the oxygen affinity all indicated that R-4 is identical with HbFu.

It may be concluded that MetHbFu is the MetHb tetramer cross-linked between β_1 82 Lys and β_2 82 Lys by a fumaryl group, and the double-headed aspirin reacted with MetHb in the same way as with Hb A.

Cross-Linking Reaction of a Valency Asymmetrical Hybrid [Hb AM ($\alpha\alpha^+\beta\beta^+$)] with the Double-Headed Aspirin

It has been suggested that in a mixture of Hb A and MetHb partial exchange of the heme moiety occurs.[21] This possibility was checked by use of HbFu and MetHbFu, both of which could not be dissociated into dimers. Thus, a mixture of 1.12 mM CO-liganded HbFu and 1.12 mM MetHbFu in 0.01 M phosphate (pH 7.4) was incubated at 37 °C for 2 h, and the mixture was analyzed by isoelectrofocusing and chromatofocusing. No bands or peaks other than those of HbFu and MetHbFu could be detected. Therefore, heme exchange did not

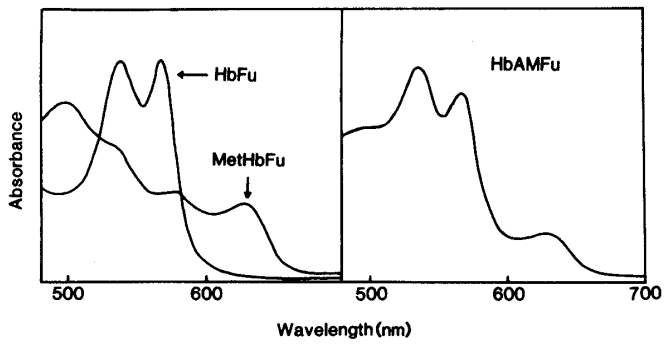

Fig. 9. Absorption Spectra of HbFu, MetHbFu and HbAMFu in 0.1 M Phosphate (pH 7.0)

All the subunits in the ferrous state were liganded with CO.

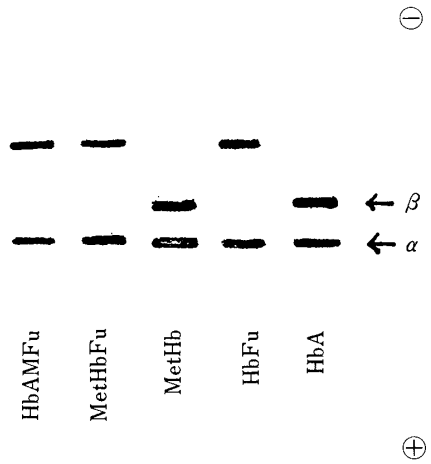

Fig. 10. SDS-PAGE of Hb A, HbFu, MetHb, MetHbFu and HbAMFu

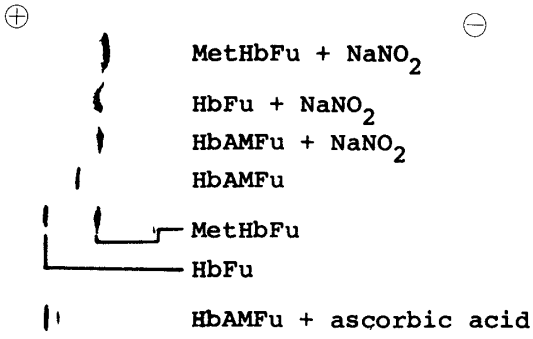

Fig. 11. Isoelectrofocusing of HbAMFu Oxidized with Nitrite and Reduced with Ascorbic Acid

occur under these conditions.

A mixture of an equimolar amount of COHb A and MetHb was incubated with the double-headed aspirin at 37 °C for 2 h. Isoelectrofocusing of the reaction mixture was performed after it had been passed through a column of Sephadex G-25 (Fig. 7). This analysis revealed that the reaction mixture was composed of four species of proteins, which can be separated and purified through chromatofocusing (A-1—A-4) (Fig. 8). The absorption spectra and isoelectric points indicated that A-1 corresponds to MetHbFu, A-2 to CO-liganded Hb A, and A-4 to CO-liganded HbFu. The fraction A-3 (HbAMFu) appeared at an intermediate position between MetHbFu and CO HbFu. In contrast to A-1 and A-4, A-3 could not be detected in the reaction mixture of Hb A or MetHb separately treated with the double-headed aspirin. Estimation of the content of met-subunit in HbAMFu from the absorption spectra (Fig. 9) revealed that half of the subunits were in met-form (52.4%). Gel filtration of HbAMFu in the presence of 1 M $MgCl_2$ revealed that it was eluted as the same position as HbFu, indicating that it is a tetrameric hemoglobin. Hb A and MetHb were split into α- and β-subunits on SDS-PAGE, and β-subunit migrated more toward the cathode than α-subunit. As was the case with HbFu and MetHbFu, HbAMFu was split into α-subunit and a component with a larger molecular weight than β-subunit (Fig. 10). These results imply that HbAMFu is the β-subunit-cross-linked tetrameric hemoglobin with half of the subunits in met-form.

When HbAMFu was oxidized with sodium nitrite, it produced MetHbFu alone on isoelectrofocusing (Fig. 11), and the absorption spectrum was identical with that of MetHb. Under the same conditions, HbFu was also converted into MetHbFu. When HbAMFu was reduced with ascorbic acid under anaerobic conditions, a major band and peak corresponding

to HbFu appeared on isoelectrofocusing (Fig. 11) and chromatofocusing. The peak from chromatofocusing corresponded to HbFu and showed an absorption spectrum similar to that of HbFu.

All these results indicated that HbAMFu is the cross-linked tetrameric hemoglobin of the valency asymmetrical hybrid [Hb AM ($\alpha\alpha^+\beta\beta^+$)] and that the cross-link was produced between β_1 82 Lys and β_2 82 Lys as in the case of HbFu and MetHbFu.

Discussion

Walder *et al.* demonstrated that double-headed aspirin [bis(3,5-dibromosalicyl)fumarate] specifically cross-links β-chains of oxyhemoglobin through a fumaryl group between the ε-amino groups of β_1 82 Lys and β_2 82 Lys in the β-cleft.[12] The β-cleft is lined with certain positively charged residues supplied by the two β chains, to which DPG binds to modulate the oxygen affinity. The double-headed aspirin with the same size as the cleft and with the negatively charged carboxylates may electrostatically interact with the cleft to form covalent cross-links and occupy the DPG binding site. The binding of the reagent is highly stereospecific to the DPG binding site of the hemoglobin molecule. We also demonstrated that the agent cross-links sickle hemoglobin [Hb S ($\alpha_2\beta_2^S$)][13] and can fix unstable asymmetrical hybrid [Hb AS ($\alpha_2\beta\beta^S$)] present in the mixture of Hb A and Hb S.[14] While Miura and Ho[15] obtained cross-linked valency asymmetrical hybrids between Hb A and Hb C, the cross-linking reaction profiles of MetHb and valency asymmetrical hybrids between Hb A and MetHb have not yet been well clarified.

In the present experiments, it was found that MetHb can be cross-linked between β_1 82 Lys and β_2 82 Lys by the reagent. Since methemoglobins have the same quaternary structure as oxyhemoglobins (R-structure),[22] it was not surprising that the reagent bound to MetHb as well as to Hb A. The reagent is useful as an intramolecular cross-linking reagent in the methemoglobin series as well as the oxy and CO hemoglobin series.[11-14]

It would be interesting to investigate the possibility of the existence of valency asymmetrical hybrid molecules in a mixture of Hb A and MetHb. Bunn and Drysdale[7] demonstrated that two kinds of intermediate are present in partially oxidized Hb A by use of the isoelectrofocusing technique. Tomoda and Yoneyama[8] showed that these two intermediates are symmetrical hybrids, ($\alpha_2^+\beta_2$) and ($\alpha_2\beta_2^+$), and found that these hybrids showed different oxygen affinity from that of Hb A.[23,24] Perutz[9] suggested that hybrids of another type, *i.e.* valency asymmetrical hybrids such as ($\alpha\alpha^+\beta_2$), ($\alpha_2\beta\beta^+$), ($\alpha\alpha^+\beta\beta^+$), ($\alpha_2^+\beta\beta^+$) and ($\alpha\alpha^+\beta_2^+$) were present as intermediates of oxidation of Hb A. If these valency asymmetrical hybrids are present, they may be readily dissociated into the unlike dimers, which may recombine with like dimers to form stable symmetrical hybrids. Perrella *et al.*[10] suggested the presence of these valency asymmetrical hybrid molecules in a mixture of Hb A and MetHb by carrying out the analysis at subzero temperature, suppressing the dissociation into dimers. In the present experiments, we fixed the valency asymmetrical hybrid [Hb AM ($\alpha\alpha^+\beta\beta^+$)] present in the mixture of Hb A and MetHb by use of the cross-linking reagent.

The following evidence indicates that the unstable valency asymmetrical hybrid [Hb AM ($\alpha\alpha^+\beta\beta^+$)] was fixed as HbAMFu, in which β_1 82 Lys and β_2 82 Lys are cross-linked through a fumaryl group. (1) HbAMFu was not formed when Hb A and MetHb were separately treated with the reagent. (2) The behavior on isoelectrofocusing of HbAMFu was intermediate between those of HbFu and MetHbFu. (3) The elution position on chromatofocusing was also intermediate between those of HbFu and MetHbFu. (4) Gel filtration in the presence of $MgCl_2$ showed that this component is a tetrameric hemoglobin which could not dissociate into dimers. (5) SDS-PAGE showed two bands corresponding to α-subunit and dimerized β-subunit, suggesting the presence of cross-linked β-subunits in this hemoglobin. (6) The

absorption spectrum of HbAMFu revealed that it contained about 50% of met-subunits. (7) Reduction of HbAMFu produced HbFu and oxidation of HbAMFu produced MetHbFu. (8) Since heme exchange was not observed under the reaction conditions, HbAMFu must be composed of $\alpha\beta$-dimer from Hb A and $\alpha^+\beta^+$-dimer from MetHb.

It is strongly suggested that the mixture of Hb A and MetHb contained unstable valency asymmetrical hybrid molecules [Hb AM ($\alpha\alpha^+\beta\beta^+$)]. Formation and dissociation of the valency asymmetrical hybrid hemoglobin may occur as depicted in Chart 2. Thus, Hb A and

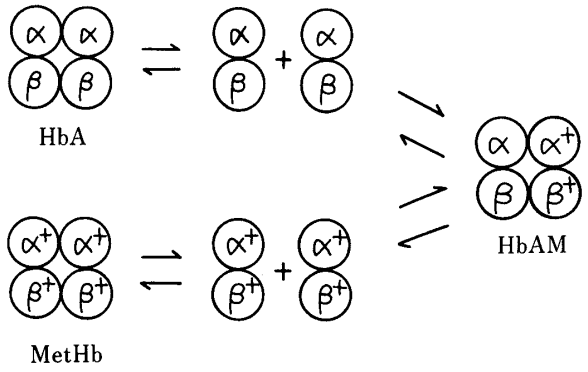

Chart 2

MetHb are dissociated into dimers,[25] and these unlike dimers can combine to form the unstable valency asymmetrical hybrid [Hb AM ($\alpha\alpha^+\beta\beta^+$)]. Hb AM can be readily dissociated into the unlike dimers, which recombine to form stable Hb A and MetHb, both having like dimer pairs. Usual analytical conditions encountered in electrophoresis and chromatography may give stable Hb A and MetHb alone, as suggested by Perutz.[9]

In conclusion, it is suggested that MetHb formed the valency asymmetrical hybrid [Hb AM ($\alpha\alpha^+\beta\beta^+$)] in the presence of Hb A. MetHb and the valency asymmetrical hybrid could be effectively cross-linked by the double-headed aspirin, as in the case with Hb A.

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