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Studies on the Intermediates of Methemoglobin by the Use of a Cross-Linking Agent

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The intermediates of methemoglobin [MetHb] were investigated by the use of an intramolecular cross-linking agent, bis(3,5-dibromosalicyl)fumarate. The cross-linked Hb A [HbFu], which cannot split into $\alpha\beta$-dimers, was oxidized with potassium ferricyanide, sodium nitrite and sesamol. In every oxidation, cross-linked hemoglobins with 1-met, 2-met, 3-met and 4-met [MetHbFu] subunits were formed. As the oxidation level increased, the contents of the hemoglobins with higher amounts of met subunits increased. MetHbFu may be produced *via* the hemoglobins with 1-met→2-met→3-met subunits. The hemoglobins with 1-met and 3-met subunits may each contain two valency asymmetrical hybrids. Whereas analysis by isoelectrofocusing showed that Hb A partially oxidized with potassium ferricyanide contained Hb A, $\alpha_2^+\beta_2$, $\alpha_2\beta_2^+$ and MetHb, all being symmetrical hybrids, treatment of the oxidized Hb A with the cross-linking agent gave corss-linked hemoglobins with 3-met subunits (valency asymmetrical hybrids) besides those with 2-met and 4-met subunits. In the course of oxidation of native Hb A, unstable valency asymmetrical hybrids may be initially produced but may split into the $\alpha\beta$-dimers which recombine and form stable valency symmetrical hybrids under analysis.

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

Methemoglobin [MetHb] ($\alpha_2^+\beta_2^+$) is produced from Hb A ($\alpha_2\beta_2$) probably *via* several intermediates. Itano and Robinson[1] suggested the presence of 3 kinds of intermediates, *i.e.* the hemoglobins with 1-met, 2-met and 3-met subunits. Other workers[2–4] demonstrated the presence of some of these intermediates by electrophoresis and column chromatography. Bunn and Drysdale[5] reported the presence of 2 kinds of intermediates by an isoelectrofocusing technique, and Tomoda and Yoneyama[6] isolated these intermediates and elucidated their structures to be $\alpha_2^+\beta_2$ and $\alpha_2\beta_2^+$, both bearing symmetrical 2-met subunits. Perutz[7] suggested that valency asymmetrical hybrids with 1-met, 2-met and 3-met subunits also exist as intermediates, and assumed that these valency asymmetrical hybrids may readily dissociate into the unlike $\alpha\beta$-dimers which recombine with like dimers to form stable symmetrical hybrids such as $\alpha_2\beta_2$ [Hb A], $\alpha_2^+\beta_2$, $\alpha_2\beta_2^+$ and $\alpha_2^+\beta_2^+$ [MetHb] under analytical conditions. Because hemoglobins tend to dissociate into $\alpha\beta$-dimers which recombine to form the stable tetramer,[8] identification of the unstable valency asymmetrical intermediate tetramers is difficult. Perrella *et al.*[9] suggested the presence of these valency asymmetrical hybrids in an analysis at subzero temperature.

Miura and Ho[10] prepared several cross-linked valency asymmetrical hybrids with 1-met, 2-met and 3-met subunits of Hb A and Hb C by the use of a cross-linking agent [bis(3,5-dibromosalicyl)fumarate].[11,12] In a previous paper,[13] we obtained a cross-linked valency asymmetrical hybrid of $\alpha\alpha^+\beta\beta^+$ [HbAMFu] in a mixture of Hb A and MetHb. This cross-linking agent provides an intramolecular cross-link between β_1 82 Lys and β_2 82 Lys, the 2,3-diphosphoglycerate binding site, with all the hemoglobins tested.[10–15] This communication deals with the valency asymmetrical intermediates of MetHb identified by the use of the cross-

linking agent.

Experimental

Absorption spectra were measured with a Shimadzu UV 200S double-beam spectrophotometer. Isoelectrofocusing was performed on Ampholine PAG plate (pH 5.5—8.5) (LKB Aminkemi) after prerunning for 1 h with a LKB 2117 Multiphor. Hemoglobins were purified by chromatography on CM-32 (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 PM 10 or PM 30 membrane (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 the use of millimolar extinction coefficients of $m\varepsilon\,569 = 13.4$[17] for CO-form, $m\varepsilon\,577 = 14.6$ for oxy-form[17] and $m\varepsilon\,630 = 4.01$[18] for met-form, on a heme basis. Isosbestic points of CO-form and met-form were found at 518 and 587 nm ($m\varepsilon\,518 = 7.15$). The content of met subunit in CO-liganded 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).

Sesamol obtained from Aldrich Chemical Company was recrystallized from chloroform–petroleum ether before use. Bis(3,5-dibromosalicyl)fumarate was prepared according to the method of Walder *et al.*[11] with slight modifications.[13] Hb A was obtained from the packed red cells from nomal human blood.[13] MetHb, cross-linked Hb A [HbFu], cross-linked MetHb [MetHbFu] and cross-linked $\alpha\alpha^{+}\beta\beta^{+}$ [HbAMFu] were prepared as described.[13]

Symmetrical Hybrids, $\alpha_2^{+}\beta_2$ and $\alpha_2\beta_2^{+}$——These hemoglobins were prepared according to the method of Tomoda and Yoneyama[19] with slight modifications. Thus, a mixture of 2.0 mM oxy Hb A and 1.0 mM potassium ferricyanide in 5.0 ml of 0.01 M phosphate (pH 7.5) was incubated at room temperature for 1 h. The reaction mixture was passed through a column of Sephadex G-25 equilibrated with 0.01 M phosphate (pH 6.8), and the hemoglobin solution was bubbled with CO gas, then applied to a column of CM-32 (1.6 × 25 cm) equilibrated with the phosphate buffer. The hemoglobins were eluted with 0.01 M phosphate (pH 7.4). The symmetrical hybrids $\alpha_2^{+}\beta_2$ and $\alpha_2\beta_2^{+}$ were obtained in CO-liganded form in yields of 20.2% and 7.5%, respectively. Isoelectrofocusing showed that they were homogeneous.

Cross-Linked $\alpha_2^{+}\beta_2$ [$\alpha_2^{+}\beta_2$Fu]——A mixture of 0.56 mM CO $\alpha_2^{+}\beta_2$ and 0.14 mM bis(3,5-dibromosalicyl)fumarate in 2.0 ml of 0.01 M phosphate (pH 7.5) was incubated at 37 °C for 2 h. The reaction mixture was desalted by passing it through a column of Sephadex G-25 equilibrared with 0.025 M Tris–HCl (pH 8.0) and bubbled with CO gas for subsequent analysis.

Oxidation of HbFu——Oxy HbFu (1.6, 2.7 and 3.4 mM) was reacted with 0.33, 1.6 and 3.1 mM potassium ferricyanide, respectively, in 0.1 M phosphate (pH 7.0) at room temperature for 1 h. Oxy HbFu (1.6, 1.2 and 3.4 mM) was treated with 0.25, 0.41 and 1.79 mM sodium nitrite, respectively, in 0.1 M phosphate (pH 7.0) at room temperature for 2 h. Oxy HbFu (2.9 mM) was treated with 2.9 mM sesamol in 0.1 M phosphate (pH 7.0) at room temperature for 15 and 90 min. Each reaction mixture was immediately passed through a column of Sephadex G-25 equilibrated with 0.025 M Tris–HCl (pH 8.0) and bubbled with CO gas for subsequent analysis.

Cross-Linking Reaction of Partially Oxidized Hb A——A mixture of 3.3 mM oxy Hb A and 1.95 mM potassium ferricyanide in 2.0 ml of 0.01 M phosphate (pH 7.5) was treated at room temperature for 10 min. The reaction mixture was immediately desalted by passing it through a column of Sephadex G-25 equilibrated with 0.01 M phosphate (pH 7.5), and the hemoglobin solution was bubbled with CO gas. The partially oxidized CO Hb A (1.8 mM), thus prepared, was reacted with 0.45 mM bis(3,5-dibromosalicyl)fumarate in 2.0 ml of 0.01 M phosphate (pH 7.5) at 37 °C for 2 h. The reaction mixture was immediately desalted by passing it through a column of Sephadex G-25 equilibrated with 0.025 M Tris–HCl (pH 8.0) for subsequent analysis.

Results

The Hb A intramolecularly cross-linked between β_1 82 Lys and β_2 82 Lys with a fumaryl group [HbFu] is not able to dissociate into $\alpha\beta$-dimers, and is a good model for investigation of the intermediates of MetHb. It has been shown that the quaternary structure of the Hb A is not greatly affected by the cross-link.[12]

HbFu was oxidized with potassium ferricyanide, sodium nitrite and sesamol. Potassium ferricyanide directly oxidizes the ferrous form of the heme moiety into ferric form.[20] Sodium nitrite oxidizes the oxy form of Hb A into MetHb with an induction period.[21] Sesamol, one of the phenolic reductants, oxidizes the oxy form of Hb A into MetHb.[22,23] Preliminary treatment of the cross-linked MetHb [MetHbFu] with the agents gave no products, indicating that the fumaryl group in the cross-linked tetramers was not affected by the agents.

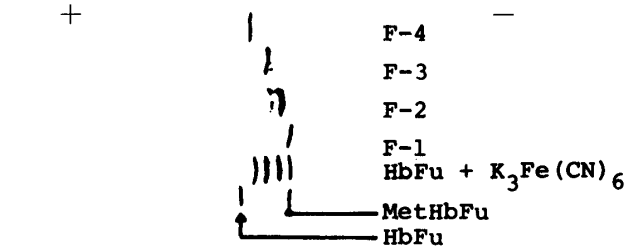

Fig. 1. Isoelectrofocusing Pattern of HbFu Oxidized with Potassium Ferricyanide

HbFu (2.7 mM) was treated with 1.6 mM potassium ferricyanide at pH 7.0 and room temperature for 1 h. The reaction mixture was immediately desalted and bubbled with CO gas. The level of heme oxidation was 61%. The products (F-1—F-4) were purified by chromatofocusing (Fig. 2).

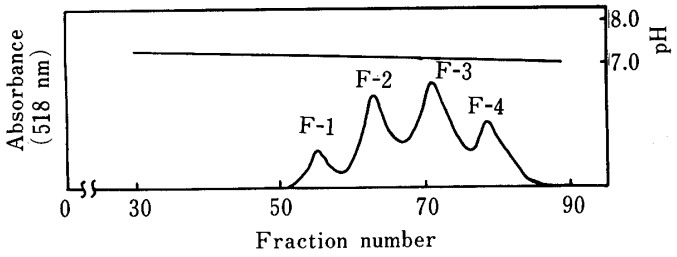

Fig. 2. Chromatofocusing Pattern of HbFu Oxidized with Potassium Ferricyanide

The reaction mixture of HbFu and potassium ferricyanide (Fig. 1) was applied to a PBE column (1.1 × 40 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. Fractions of 2.0 ml were collected.

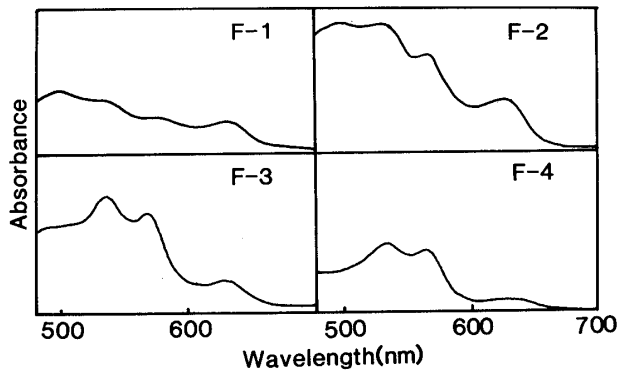

Fig. 3. Absorption Spectra of the Purified Products (F-1—F-4) in HbFu Oxidized with Potassium Ferricyanide

Spectra were taken in 0.1 M phosphate (pH 7.0). The heme moiety in ferrous form was liganded with carbon monooxide.

The oxy form of HbFu was oxidized with 0.6 eq of ferricyanide at pH 7 and room temperature for 1 h. The products were converted into CO form. Spectrophotometric estimation of the hemoglobins showed that 61% of the hemoglobins had been oxidized. Isoelectrofocusing analysis of the products revealed that they were composed of at least four products (Fig. 1), which were separated and purified by chromatofocusing (Fig. 2). The product F-1 focused at the same position of MetHbFu, and the products F-2, F-3 and F-4 focused at intermediate positions between HbFu and MetHbFu, with higher isoelectric points in that order. The product F-3 focused at the same position as the standard cross-linked hemoglobins with 2-met subunits, HbAMFu[13] and $\alpha_2^+\beta_2$ Fu. Absorption spectra (Fig. 3) indicated that F-1 contained 94.3% met subunit; F-2, 72.7%; F-3, 55.3%; and F-4, 30.9%. It was concluded that F-1 was MetHbFu, F-2 HbFu with 3-met subunits, F-3 HbFu with 2-met subunits and F-4 HbFu with 1-met subunit.

Oxidation of the oxy form of HbFu with 0.2 eq of potassium ferricyanide gave products with 19% met subunits. Isoelectrofocusing and chromatofocusing indicated that the products were composed of unoxidized HbFu and HbFu (1-met subunit). Oxidation of the oxy form of HbFu with 0.9 eq of potassium ferricyanide gave products with 85% met subunits. The products were composed of HbFu (2-met subunits), HbFu (3-met subunits) and MetHbFu.

The yields of the cross-linked Hb with different contents of met subunits were estimated from the absorbance at 518 nm, and the results are summarized in Table I. As the oxidation level increased, the contents of the HbFu with higher content of met subunits increased. Thus, the oxidation of HbFu with potassium ferricyanide appears to proceed *via* the hemoglobins with 1-met → 2-met → 3-met → 4-met subunits.

TABLE I. Distribution of Intermediate Hemoglobins in Partially Oxidized HbFu

Oxidant	Equivalent to HbFu	Content of met subunits (%)	Percentages of cross-linked products				
			0-met	1-met	2-met	3-met	4-met
Potassium ferricyanide	0.20 [a]	19	42	58	0	0	0
	0.60 [a]	61	0	24	33	31	12
	0.80 [a]	85	0	0	26	34	40
Sodium nitrite	0.18 [b]	28	33	67	0	0	0
	0.35 [b]	63	0	15	39	26	20
	0.55 [b]	82	0	0	25	37	37
Sesamol	1.00 [c]	38	10	35	54	0	0
	[d]	80	0	0	35	29	36

The oxy form of HbFu at a concentration between 1 and 4 mM was treated with the agent at pH 7.0 and room temperature for *a*) 1 h, *b*) 2 h, *c*) 15 min and *d*) 90 min. The peaks that appeared on chromatofocusing (Fig. 2) were determined to be 0-met [HbFu] (the peak after F-4); 1-met (F-4); 2-met (F-3), 3-met (F-2) and 4-met [MetHbFu] (F-1). Percentages of the products were determined from the absorbance values at 518 nm.

TABLE II. Distribution of Intermediate Hemoglobins in the 76%-Oxidized Hb A Uncross-Linked and Cross-Linked by Bis(3,5-dibromosalicyl)fumarate

	Percentages of the intermediates				
	0-met	1-met	2-met	3-met	4-met
76%-Oxidized Hb A	6	0	42	0	52
76%-Oxidized and cross-linked Hb A	0	0	38	37	24

Hb A was oxidized with potassium ferricyanide and the products were separated by CM-32. The contents of 0-met [Hb A], 2-met [$\alpha_2^+\beta_2 + \alpha_2\beta_2^+$] and 4-met [MetHb] were determined from the absorbance values at 518 nm. The partially oxidized Hb A was treated with bis(3,5-dibromosalicyl)fumarate, and the cross-linked components 2-met (F-5′), 3-met (F-4′) and 4-met (F-3′) [MetHbFu] separated by chromatofocusing (Fig. 5) were determined spectrophotometrically.

The oxy form of HbFu was treated with 0.18, 0.35 and 0.55 eq of sodium nitrite at pH 7 and room temperature for 2 h. Spectrophotometric estimation of the products showed that the contents of the met subunit were 28%, 63% and 82%, respectively. Separation of the products by chromatofocusing and subsequent analysis by isoelectrofocusing and spectrophotometry showed that the 28%-oxidized HbFu contained HbFu and HbFu (1-met subunit), the 63%-oxidized HbFu contained HbFu (1-met subunit), HbFu (2-met subunits), HbFu (3-met subunits) and MetHbFu, and the 82%-oxidized HbFu contained HbFu (2-met subunits), HbFu (3-met subunits) and MetHbFu. The oxy form of HbFu was treated with an equivalent amount of sesamol at pH 7 and room temperature for 15 and 90 min. Spectrophotometric estimation of the products showed that met subunit contents in the products after 15 min and 90 min were 38% and 80%, respectively. Separation of the products by chromatofocusing and subsequent analysis by spectrophotometry indicated that the 38%-oxidized HbFu contained HbFu, HbFu (1-met subunit) and HbFu (2-met subunit), and the 80%-oxidized HbFu contained HbFu (2-met subunits), HbFu (3-met subunits) and MetHbFu. Thus, treatment of the oxy form of HbFu with sodium nitrite and sesamol gave three intermediates with different oxidation states. As the oxidation level increased, the contents of the hemoglobins with higher amounts of met subunits increased (Table I). This indicates that the oxidation proceeds *via* the hemoglobins with 1-met→2-met→3-met subunits (Chart 1).

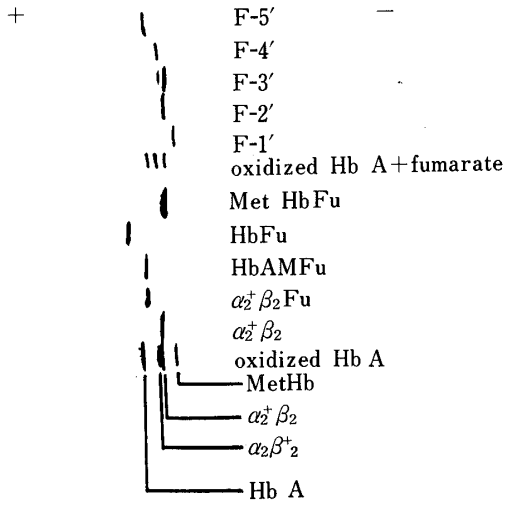

Fig. 4. Isoelectrofocusing Pattern of Hb A Oxidized with Potassium Ferricyanide and Its Products Cross-Linked by Bis(3,5-dibromosalicyl)fumarate

Hb A (3.3 mM) was treated with 1.95 mM potassium ferricyanide at pH 7.5 and room temperature for 10 min. The 76%-oxidized Hb A thus prepared (1.8 mM) was reacted with 0.45 mM bis(3,5-dibromosalicyl)fumarate at pH 7.0 and 37 °C for 2 h. The reaction products (F-1′—F-5′) were purified by chromatofocusing (Fig. 5). The symmetrical hybrid $\alpha_2^+\beta_2$ was obtained from the partially oxidized Hb A,[19] and its cross-linked derivative was prepared by reaction with bis(3,5-dibromosalicyl)fumarate. MetHbFu, HbAMFu and HbFu were obtained as described.[13]

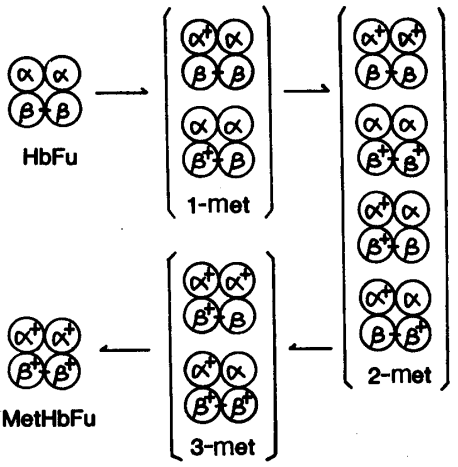

Chart 1

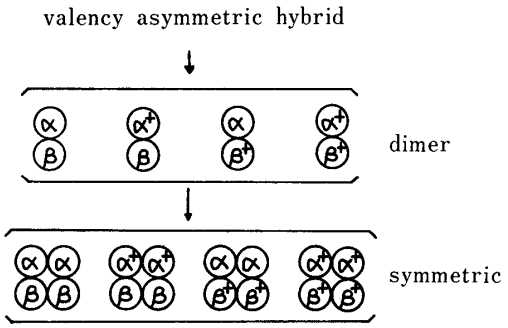

Chart 2

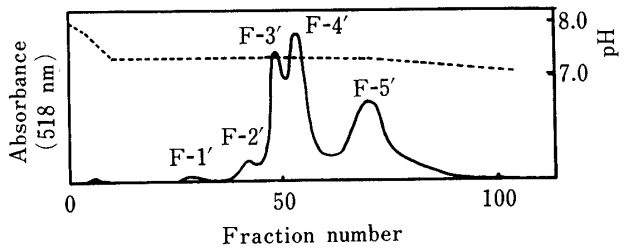

Fig. 5. Chromatofocusing Pattern of the Reaction Mixture of Partially Oxidized Hb A and Bis(3,5-dibromosalicyl)fumarate

The reaction mixture of partially oxidized Hb A and bis(3,5-dibromosalicyl)fumarate (Fig. 4) was applied to a PBE column (1.6 × 27 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. Fractions of 2.0 ml were collected.

The oxy form of Hb A was oxidized with 0.6 eq of potassium ferricyanide at pH 7.5 and room temperature for 10 min. Spectrophotometric estimation indicated that the content of met subunit in the oxidized hemoglobins was 76%. When the partially oxidized Hb A was analyzed by isoelectrofocusing, four bands corresponding to Hb A, $\alpha_2^+\beta_2$, $\alpha_2\beta_2^+$ and MetHb were observed (Fig. 4), which is in accordance with the result obtained by Tomoda and Yoneyama.[6,19] The distribution of these components was determined after separation by CM-32 column chromatography[19] (Table II).

The 76%-oxidized Hb A was treated with 0.25 eq of bis(3,5-dibromosalicyl)fumarate at pH 7.5 and 37 °C for 2 h. Analysis by isoelectrofocusing revealed three major bands (Fig. 4). The products were separated into 5 peaks (F-1′—F-5′) by chromatofocusing (Fig. 5), and the purified products were analyzed by isoelectrofocusing (Fig. 4). Since the cross-linking reaction by bis(3,5-dibromosalicyl)fumarate was not complete under these conditions,[11-15] uncross-linked tetramers may exist in the reaction mixture. Standard uncross-linked hemoglobins Hb A, $\alpha_2\beta_2^+$, $\alpha_2^+\beta_2$ and MetHb focused at more acidic positions in that order, and the stan-

dard cross-linked hemoglobins HbFu,[13] $\alpha_2^+\beta_2$ Fu and MetHbFu[13] focused at more acidic positions than those of the corresponding uncross-linked hemoglobins because of the blocking of the ε-amino groups at β_1 82 Lys and β_2 82 Lys. HbAMFu, cross-linked valency asymmetrical hybrid,[13] focused at the same position as $\alpha_2^+\beta_2$ Fu. The minor product F-1′ focused at the same position as MetHb. The products F-2′ and F-3′ focused at the same position as $\alpha_2^+\beta_2$ and MetHbFu. The product F-4′ focused at an intermediate position between MetHbFu and HbAMFu. The product F-5′ focused at the same position as HbAMFu and $\alpha_2^+\beta_2$ Fu. Spectrophotometric analysis of each product indicated that F-1′ contained 4-met subunits; F-2′ 2-met subunits; F-3′ 4-met subunits; F-4′ 3-met subunits; and F-5′ 2-met subunits. The minor products F-1′ and F-2′ were identified as MetHb and $\alpha_2^+\beta_2$, respectively, by spectrophotometric analysis and from the isoelectric points. Other products were found to be cross-linked hemoglobins since they were eluted at the tetramer position in gel filtration through an Ultrogel AcA 44 column equilibrated with 1 M $MgCl_2$[13] (data not shown). Thus, the product F-3′ was identified as MetHbFu, F-4′ as HbFu (3-met subunits), and F-5′ as HbFu (2-met subunits). The distribution of these cross-linked hemoglobins is shown in Table II.

It is interesting that whereas the 76%-oxidized Hb A produced hemoglobins with 0-met, 2-met and 4-met subunits on isoelectrofocusing and chromatography, the cross-linking reaction of the partially oxidized Hb A provided cross-linked hemoglobins with 3-met subunits besides those with 2-met and 4-met subunits. It is likely that hemoglobins with 3-met subunits exist as unstable intermediates of MetHb. It may be concluded that the oxidation of Hb A proceeds *via* processes involving intermediates other than the hemoglobins with 2-met subunits.

Discussion

It is assumed that the intermediates with 1-met, 2-met and 3-met subunits are produced in the course of the formation of MetHb from Hb A.[7] These intermediates included valency symmetrical hybrids such as $\alpha_2^+\beta_2$ and $\alpha_2\beta_2^+$, and valency asymmetrical hybrids such as $\alpha\alpha^+\beta_2$, $\alpha_2\beta\beta^+$, $\alpha\alpha^+\beta\beta^+$, $\alpha\alpha^+\beta^+\beta$, $\alpha_2^+\beta\beta^+$ and $\alpha\alpha^+\beta_2^+$. Tomoda and Yoneyama demonstrated the presence of valency symmetrical hybrids as intermediates of MetHb but observed no other intermediates.[6,19] Perutz[7] suggested that the valency asymmetric hybrids are unstable and readily split into the αβ-dimers under analytical conditions such as electrophoresis and chromatography. The dimers may recombine with like dimers to form stable symmetrical tetramers such as Hb A, $\alpha_2^+\beta_2$, $\alpha_2\beta_2^+$ and MetHb (Chart 2). In the previous study,[13] we obtained the cross-linked valency asymmetrical hybrid of $\alpha\alpha^+\beta\beta^+$ [HbAMFu] in a mixture of purified Hb A and purified MetHb, and demonstrated the presence of the valency asymmetrical hybrid $\alpha\alpha^+\beta\beta^+$.

In the present communication we describe the oxidation of HbFu by potassium ferricyanide, sodium nitrite and sesamol. HbFu is the hemoglobin cross-linked at β_1 82 Lys and β_2 82 Lys by the fumaryl group, and its conformation is very similar to that of native Hb A.[12] The cross-linked Hb A is a good model for the study of the intermediates of MetHb since it does not split into αβ-dimers. Partially oxidized HbFu contained three kinds of intermediates of MetHbFu. They were found to be HbFu with 1-met subunit, 2-met subunits and 3-met subunits. As the oxidation level increased, the hemoglobins with higher contents of met subunits increased (Table I). MetHbFu was formed *via* the hemoglobins with 1-met subunit→2-met subunits→3-met subunits (Chart 1). HbFu with 1-met subunit and with 3-met subunits may each be composed of at least two valency asymmetrical hybrids. HbFu with 2-met subunits may contain two valency symmetrical hybrids and two valency asymmetrical hybrids. It is suggested that valency asymmetrical hybrid hemoglobins are produced as

intermediates of MetHb.

The cross-linking reaction of the 76%-oxidized HbA by bis(3,5-dibromosalicyl)fumarate afforded HbFu with 3-met subunits besides HbFu with 2-met subunits and MetHbFu. Whereas the partially oxidized and uncross-linked Hb A was separated into 0-met [Hb A], 2-met [$\alpha_2^+\beta_2 + \alpha_2\beta_2^+$] and 4-met [MetHb], all being valency symmetrical hybrids, on isoelectrofocusing and chromatography, the partially oxidized and cross-linked Hb A contained no 0-met [HbFu], but did contain 2-met, 3-met and 4-met. This result demonstrates that valency asymmetrical hybrids may be present as intermediates of MetHb.

The present results support the proposal of Perutz,[7] who suggested the presence of valency asymmetrical hybrids as intermediates of MetHb, and agree with the results obtained by Perrella *et al.*[9] who observed valency asymmetrical hybrids as intermediates of MetHb in an analysis at subzero temperature. Under usual analysis conditions of the intermediates of MetHb by isoelectrofocusing and chromatography,[6,19] unstable asymmetrical hybrids may split into the unlike $\alpha\beta$-dimers which may readily recombine with like dimers to form stable symmetrical tetramers such as Hb A, $\alpha_2^+\beta_2$, $\alpha_2\beta_2^+$ and MetHb.

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