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REACTION OF HAPTOGLOBIN WITH ISOLATED HEMOGLOBIN CHAINS

BY

Yasuo SUGATA*1

ABSTRACT

Isolated hemoglobin chain, namely α- or β-chain, with sulfhydryl groups masked with p-chloromercuribenzoate, and $\alpha\beta$ dimer partly masked with p-chloromercuribenzoate were tested for their reactivity with haptoglobin.

The α-chain reacts with haptoglobin but it was excluded from α-chain haptoglobin complex by the successibly added native hemoglobin. The β-chain does not bind with haptoglobin without α-chain, but readily binds in the presence of α-chain to form a sufficiently stable complex. The $\alpha\beta$ dimer masked with its reactive sulfhydryl group seems to bind with haptoglobin as does native hemoglobin. These facts seem to be an evidence that haptoglobin reacts with hemoglobin dimer as a unit and suggest that unit dimer for this reaction is α^1-β^1 dimer rather than α^1-β^2 dimer.

INTRODUCTION

Haptoglobin (Hp), a serum glycoprotein, binds with hemoglobin (Hb) stoichiometrically over a wide range of pH to give a very stable complex[1]. In the case of human Hp 1–1 and the Hp of other vertebrates, the complex consists of one molecule of Hp and one molecule of Hb (Hb-Hp), when it is generated in the presence of excess Hb, but when less than an equivalent amount of Hb is added, an intermediate complex which consists of one molecule of Hp and one-half molecule of Hb ($\alpha\beta$-Hp) is formed[2–4]. The reaction between Hp and Hb involves one-half molecule of Hb[5–9].

Haptoglobin of any species readily combine with liganded hemoglobins of a number of other vertebrate species[1], which deoxy Hb and Hb H binds very little[10,11], if at all, and Hb treated with bis (*N*-maleimidomethyl) ether shows impaired binding with Hp[5]. These molecules have decreased degree of dissociation. On the other hand, even in a deoxygenated state, carboxypeptidase A-digested Hb binds with Hp[11], and this seems to be correlated to the absence of related conformational changes in the deoxygenated form[12]. Thus, Hp seems to require Hb to split into dimers. Iso-

*1 菅田安男: Department of Clinical Genetics (Chief: Prof. H. NAKAJIMA), School of Medicine, Tokyo Medical and Dental University (Tokyo Ika Shika Daigaku).
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lated Hb chain with free sulfhydryl (SH) groups or with SH-groups blocked with p-chloromercuribenzoate (PCMB) binds with Hp irrespective of presence of ligands[11]. It has been pointed out that sole β-chain has a low affinity and its reactivity with Hp depends on its concentration, but the α-chain facilitates the reactivity of β-chain[7,13]. Studies with isolated Hb chain suggested that Hp has four binding sites for a monomeric chain. In this case, also, Hp seems to require Hb to form a unit dimer of unlike subunits on the Hp molecule.

On the other hand, Hb consists of four subunits, and dissociation of tetramer to dimer exposes its interface with which subunits are associated. The Hb tetramer is in dynamic equilibrium with the dimers and monomers. Symmetrical dissociation into dimers of type $\alpha_2\beta_2 \rightleftharpoons 2\,\alpha\beta$ takes place at a high concentration of neutral electrolite or of urea, but asymmetrical dissociation into subunits of the type $\alpha_2\beta_2 \rightleftharpoons \alpha_2 + \beta_2$ is not observed[14]. The planes along which Hb may dissociate into symmetrical dimer were identified by using a specific nature of the reaction with PCMB. According to Perutz[15], the longer distance (30.4 Å) is represented for α^1–β^1 and a shorter one (25.2 Å) for α^1–β^2 *2. PCMB attaches at position β-93 which is located near the contact of α^1 and β^2, prior to initial dissociation to $\alpha\beta$-dimer, and further dissociation to monomers by PCMB does not occur with hemoglobins which do not contain SH-groups on the β-112 near the contact α^1 and β^1. These findings suggest that the Hb dimer obtained by treating with PCMB is α^1–β^1 dimer[16].

Two questions now arise from these observations. The first one is which side of surface on the Hb dimer molecule attaches to Hp, the interface that is exposed by dissociation to a dimer or the outside of the original tetrameric Hb. The second question is which of the two possible dimers attaches to Hp in the case of Hb-Hp generation, α^1-β^1 or α^1-β^2 (according to Perutz's designation), because naturally occuring dimer is thought to be α^1-β^1 as a major possibility[16,17], α^1-β^2 dimer is proposed as another possibility[18].

In the present study, α- and β-chains for monomer subunits and the mixture of β_2 and $\alpha\beta$ chains for dimer subunits were examined for their ability to bind with Hp, and stability of the complex with such isolated chain was examined by replacement reaction with ^{14}C-labeled Hb.

Available evidence suggests that Hb binds to Hp with dimer as a unit. The experiment on the stability of the Hb-Hp complex generated from unlike subunits even when masked with their SH-groups by PCMB indicates that the unit is α^1-α^1 dimer rather than α^1-β^2 dimer.

*2 The numbers are written as superscripts to avoid confusion with formulae for the molecular forms in which the subunits are associated, e.g. $\alpha_2\beta_2$ for the tetramer, $\alpha_1\beta_1$ and β_2 for the dimer, and α_1 and β_1 for the monomer.

Materials and Methods

(a) Preparation of Hb A_1

Hb A_1 was purified from human hemolysate by the chromatography on DEAE-Sephadex column washed with 0.01 M potassium phosphate buffer (PPB), pH 8.5, and eluted with 0.2 M NaCl.

(b) Preparation of isolated Hb chains

Isolated Hb chains were prepared by the method of Geraci[19] from purified Hb. PCMB-treated Hb A_1 was separated to two fractions by chromatography on CM-cellulose column equilibrated with 0.01 M PPB, pH 6.5. The first fraction was obtained from the column by eluting with 0.01 M PPB, pH 6.5.

Then the second fraction was obtained by eluting with 0.1 M NaCl. The first fraction was further separated to two fractions by gel filtration through Sephadex G-100 column. The fast and slow filtrating fractions were named as fraction 2 (Fr. 2) and fraction 1 (Fr. 1), respectively. β_4-Tetramer was regenerated from Fr. 1 by removal of PCMB using overnight dialysis against 0.05% thioglycol in 0.05 M PPB, pH 7.0.

(c) Estimation of free sulfhydryl groups

Sulfhydryl titrations were carried out according to the method of Boyer[20]. PCMB was determined spectrophotometrically by the use of a value of 16.9 as the millimolar extinction in 0.05 M PPB, pH 7.0, at 232 mμ. Resulting mercaptide was tentatively estimated by the use of a value of 12.8 as the millimolar extinction at 250 mμ. Three milliliter of Hb in about 0.05 mM concentration was titrated with increment of about 0.13 mM PCMB. Hb was determined spectrophotometrically using millimolar extinction of 14.3 at 542 mμ or 25.7 at 250 mμ for HbO_2. Correction for metification of Hb was made by calculating metHb content from the following equation. At pH 7.0, HbO_2 has an extinction coefficient of 14.3 $mM^{-1}cm^{-1}$ at 542 mμ nd 5.1 at 500 mμ. And that of metHb is 6.0 and 9.2 respectively.

$$\text{metHb } (\%)=\frac{14.3-5.07r}{7.05+6.42r}\times 100, \quad r=\frac{E_{542m\mu}}{E_{500m\mu}}$$

(d) Determination of molecular weight of Hb chains

It was obtained by gel filtration through Sephadex G-100 column. The column size was 65×2.5 cm and the running buffer was 0.05 M PPB, pH 7.0. The samples were collected in 3.0 ml fractions. The volume of applied protein mixture was 1.0 ml. A semilogarithmic plot of the molecular weight *versus* K_{av}[31] gives the molecular weight of the samples graphically. Standard proteins purchased from Mann are ovalbumin (molecular weight;

45,000), beef pancreatic chymotrypsinogen A (25,000) and horse heart cytochrome c (12,400). Blue dextran 2000 (Pharmacia) was used for estimation of void volume.

(e) Starch gel electrophoresis

Thin layer starch gel electrophoresis was carried out at pH 8.6 according to the method of Smithies[22] with some modifications. For gel preparation, 15% hydrolysed starch (Connaught) in Tris-EDTA-borate buffer was used. And 0.3 M borate buffer was used for electrode vessels. Operating currents were about 10 mA/cm^2.

(f) Preparation of Hp

Horse Hp was prepared at pH 7.0 throughout the procedure according to the method of Hamaguchi[23] with minor modification.

(g) Preparation of labeled Hb

Hb labeled with ^{14}C was prepared by incubating of human red cells from a patient of hemolytic anemia which contain about 9% of reticulocytes in Borsook's medium described by Vinograd and Hutchinson[25] containing leucine uniformly labeled with ^{14}C. This labeled Hb was counted in a gas flow counter (Aloka). Specific activity of Hb was 200 counts/min./mg Hb.

(h) Standard method of studying reactivity of Hp with Hb chains

Mixed sample of 1.7 mg (expressed as Hb binding capacity) of Hp and 1.9 mg of Hb or isolated Hb chain with or without α-chain was applied to Sephadex G 100 column (65×2.5 cm) equilibrated with 0.05 M PPB,

Fig. 1. Electrophoretic patterns of Hb A_1 and dissociated Hb A_1. Starch gel analyses in a Tris-EDTA-borate buffer, pH 8.6, without PCMB.
(i), (v) Purified Hb A_1
(ii) Dissociated Hb A_1 by treating with near equivalent PCMB per SH group.
(iii) The fraction separated from (ii) by chromatography of CM-cellulose by the use of 0.01M PPB, pH 6.5.
(iv) α-Chain, obtained as the second eluate by the use of 0.2M NaCl from the column same as (iii).

Fig. 2. Electrophoretic patterns of Fr. 1 and Fr. 2. Analytical conditions are the same as those in Fig. 1.
(i) The eluate from the CM-cellulose column, same as (iii) in Fig. 1.
(ii) Fr. 1, which is the slower peak of filtrate of (i) through Sephadex G-100,
(iii) Fr. 2, which is the faster peak of filtrate of (i) through Sephadex G-100.

Fig. 3. Electrophoretic patterns of SH-group -regenerated Fr. 2. Analytical conditions are the same as those in Fig. 1.
(i) The eluate from CM-cellulose column, same as (iii) in Fig. 1.
(ii) SH group-regenerated Fr. 2 by dialysis against 0.05% thioglycol in 0.05M PPB, pH 7.0.
(iii) Purified Hb A_1.

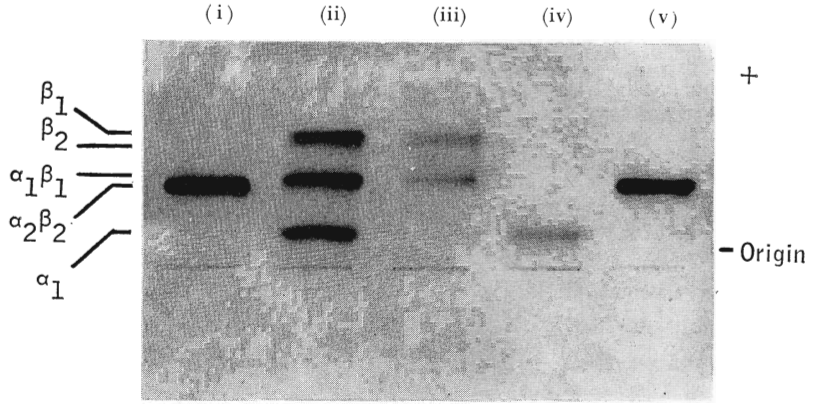

Fig. 1

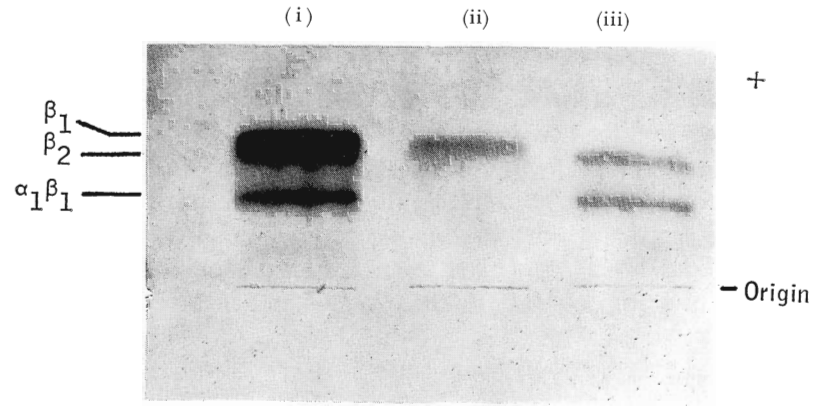

Fig. 2

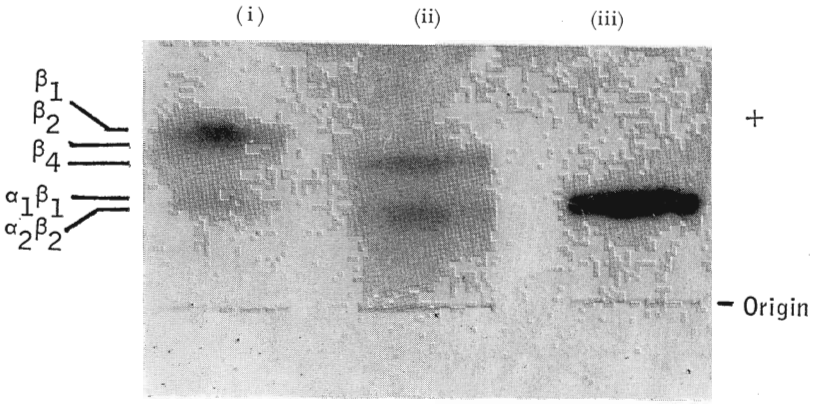

Fig. 3

pH 7.0. Hemoglobin-haptoglobin complex (Hb-Hp) (filtrated at fraction number around 37) (see Figs. 5–9), Hb (filtrated at fraction number around 54), and isolated Hb chain (filtrated at fraction number around 65 as dimer and at around 78 as monomer) were monitored by determining the absorbancy at 414 mμ for HbO_2. The reactivity of Hp with Hb chain was compared with each other by the altitude of complex region in the gel filtration pattern. Another proof of reactivity has been performed using ^{14}C-cyanmetHb. In order to clarify the reactivity and the stability of Hb-Hp generated from examined Hb or Hb chains, the samples mixed with Hp were added by labeled Hb. The filtration pattern was obtained by determining Hb as cyanmet-form at 421 mμ, and each 2.5 ml of the fraction was dried on a planchet and was counted its radioactivity by a gas-flow counter for 20 minutes. The specific activity was expressed as,

$$\text{Specific activity} = \frac{\text{counts per 10 minutes}}{\text{extinction at 421 m}\mu \times 10^3}$$

In this experiment, if previously bound Hb chain is stable enough, the decrease in the specific activity at complex region in the filtration pattern would be observed. If previously bound Hb chain is less stable, it would be expected that labeled Hb replaces the Hb chain from its complex with Hp and the specific activity of complex would remain unchanged from that of labeled Hb-Hp. Moreover, excluded Hb chain would be found as increased altitude of original Hb chain region in gel filtration pattern.

Results

(a) Starch gel electrophoretic patterns of PCMB-treated Hb

All six components appeared in Figs. 1–3 were interpreted as α_1-, $\alpha_2\beta_2$-, $\alpha_1\beta_1$-, β_4-, β_2-, and β_1 hemoglobins according to their mobility from cathode to anode. The $\alpha_1\beta_1$ and $\alpha_2\beta_2$ seemed to differ in their electrophoretic mobility according to their molecular size. A small difference in migration rate was also found in the case of β_1, β_2, and β_4 hemoglobins. Electrophoretic patterns of purified Hb A_1(i) and PCMB-treated Hb A_1(ii) are presented in Fig. 1. Hb A_1 treated with equimolar PCMB as SH-groups was separated into three major components, namely β-chain (β_1 and β_2) $\alpha\beta$-chain ($\alpha_1\beta_1$), and α-chain. The PCMB-treated Hb A_1 was separated by chromatography on CM-cellulose column equilibrated with 0.01 M PPB, pH 6.5. The first fraction eluted from CM-cellulose column with 0.01 M PPB, pH 6.5(iii) is consisted of two β-chains (β_1 and β_2) and $\alpha_1\beta_1$ chain which migrates slightly faster than $\alpha_2\beta_2$. The second fraction eluted from CM-cellulose column with 0.1 M NaCl was α-chain(iv). Fr. 2 (Fig. 2, iii) and

Fr. 1 (Fig. 2, ii) which are separated by gel filtration through Sephadex G-100 column, seemed to correspond to dimer containing $\alpha_1\beta_1$ and β_2 and monomer containing β_1 only, respectively. When Fr. 2 was dialysed against 0.05% thioglycol in 0.05 M PPB, pH 7.0, overnight, the migration rate was observed to decrease a little compared with its parent material due to the formation of tetramer (Fig. 3, ii). Tetramer generation has been chequed by gel filtration through Sephadex G-100 column.

(b) Estimation of molecular weight of Fr. 1, Fr. 2, and α chain

From a semilogarithmic plot of the molecular weight *versus* K_{av}, molecular weights of the Fr. 1 (β_1-chain), Fr. 2 (β_2 and $\alpha\beta$ dimer), and α chain were assumed to be 17,000, 33,000, and 16,000, respectively (Fig.)4).

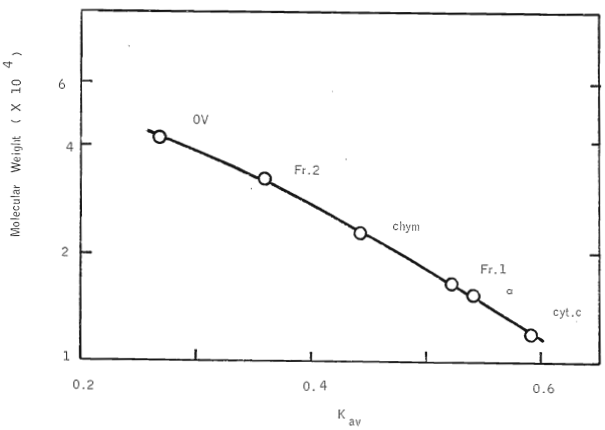

Fig. 4. Graphical estimation of molecular weight of Fr. 1, Fr. 2 and α-chain.
A plot of the molecular weight *versus* K_{av}.
ov: ovalbumin, chy: beef pancreatic chymotrypsinogen A, cyt. c: horse heart cytochrome c.

(c) Determination of free sulfhydryl groups of isolated Hb chains

No free SH-groups were found in either Fr. 1 or Fr. 2 (Table 1). The contents of PCMB in the two fractions were calculated from the basis of millimolar extinction of 12.8 for mercaptide formed and 25.7 for Hb in heme at 250 mμ. The number of free SH-groups was in good agreement with the earlier report[26] for Hb A and regenerated β-chain. From the data of the rough estimation of bound PCMB, β_4 indicates its incompleteness of regeneration. Fraction 1 seems to be completely saturated or over-saturated by PCMB, while Fr. 2 seems to have masked SH-groups because at least 1.5 of total SH-groups per heme are expected from the composition of Fr. 2 which consists of β_2 and $\alpha\beta$ dimer.

(d) Reactivity of Hp with isolated Hb chains

Table 1. Estimation of free SH-groups and tentative estimation of bound PCMB

Sample	Free SH/heme	$E^{mM}_{Hb,\ 250m\mu}$[1]	$E_{PCMB,\ 250m\mu}$[2]	Bound PCMB/heme[3]
Hb A_1	0.42	24.7	0.0	0.0
β_4[4]	1.89	28.7	4.0	0.31
α-chain	0.07	34.1	9.4	0.73
Fr. 1	0.0	59.6	39.9	2.70
Fr. 2	0.0	35.1	10.4	0.81

1 Millimolar extinction of Hb calculated from the ratio of extinction at 542 mμ to 250 mμ.
2 Extinction of PCMB-mercaptide per heme, calculated from the difference between $E^{mM}_{Hb,\ 250m\mu}$ and the value of 24.7, the millimolar extinction of Hb A.
3 The value of $E_{PCMB,\ 250m\mu}$ divided by 12.8, which is the millimolar extinction of PCMB-mercaptide at 250 mμ.
4 Regenerated from Fr. 1.

Reactivity of Hp with isolated Hb chain was examined by the standard method described above. Figs. 5 to 9 are indicated as superimposed chart from different runs with or without labeled Hb. Complex formation was observed as an increase in absorbance of the fractions around fraction number 37.

The α-chain reacted with Hp (Fig. 5), however, it was replaced by

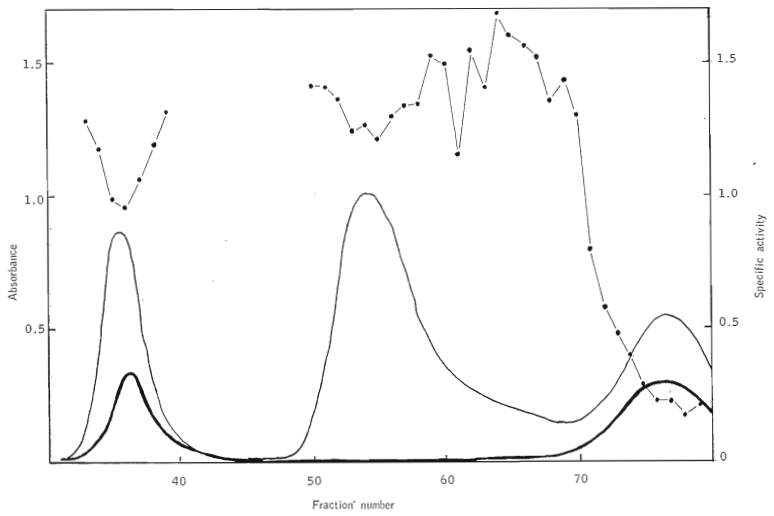

Fig. 5. Gel filtration patterns of the mixture of Hp and α-chain, with or without ^{14}C-labeled Hb
———— : Experiment without labeled Hb. Absorbance was determined at 414 mμ as HbO_2.
———— : Experiment with labeled Hb. Absorbance was determined at 421 mμ as cyanmetHb.
—●—●— : Specific activity (see text for its definition).

labeled Hb from α-chain-Hp complex after the addition of labeled Hb. Thus, the specific activity of the complex was as high as that of the labeled Hb-Hp complex (Table 2), and the amount of α-chain recovered up to the level of original α-chain without Hp (Table 2, last column).

In the case of Fr. 1 which consists of β_1-chain, sole β-chain could not react with Hp, and Hp was saturated with labeled Hb (Fig. 6, fraction

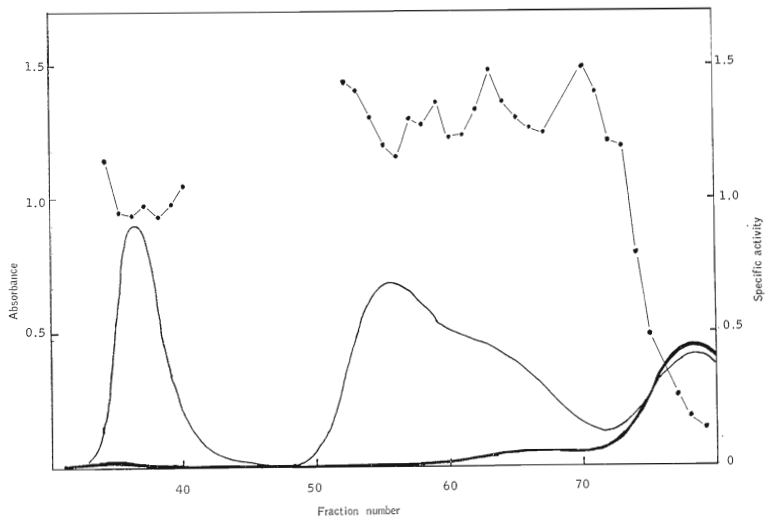

Fig. 6. Gel filtration patterns of the mixture of Hp and Fr. 1. Legends same as in Fig. 5.

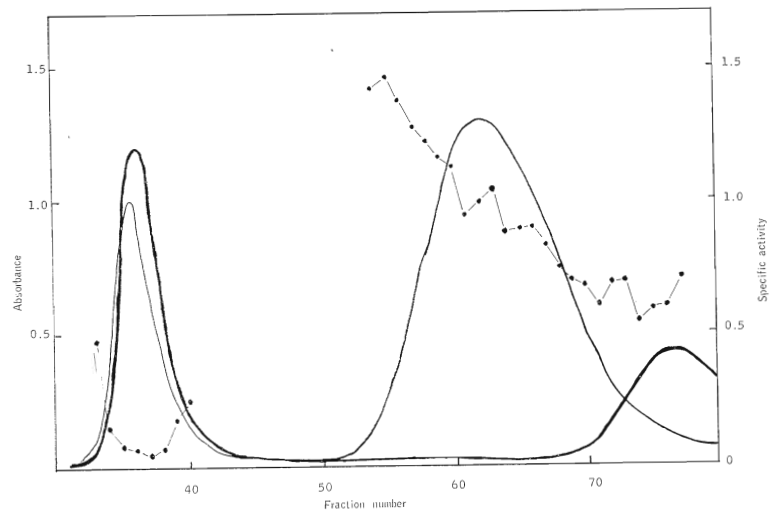

Fig. 7. Gel filtration patterns of the mixture of Hp, Fr. 1, and α-chain. Legends same as in Fig. 5.

number around 37), and the amount of β-chain remained unchanged (Fig. 6, fraction number around 77). On the other hand, β-chain readily reacted with Hp in the presence of α-chain (Fig. 7). The decreased specific activity as low as 0.05 shows that the complex resulting from α-chain and β-chain masked with PCMB is not replaced by labeled Hb and is as stable as the

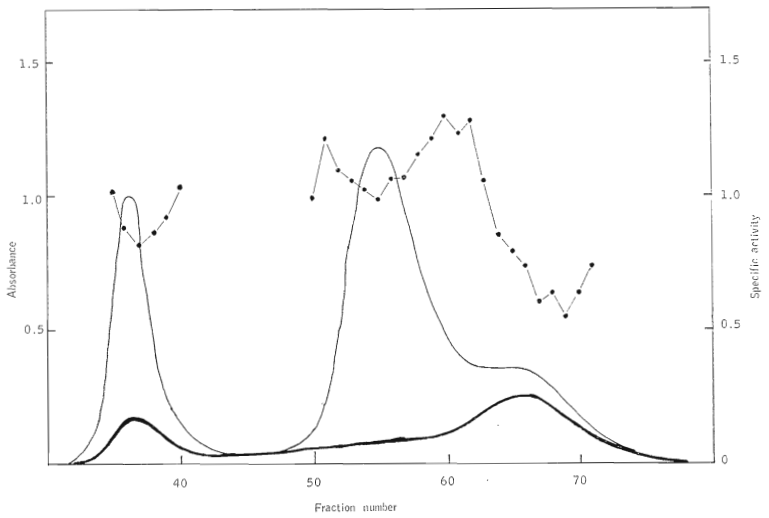

Fig. 8. Gel filtration patterns of the mixture of Hp and Fr. 2. Legends same as in Fig. 5.

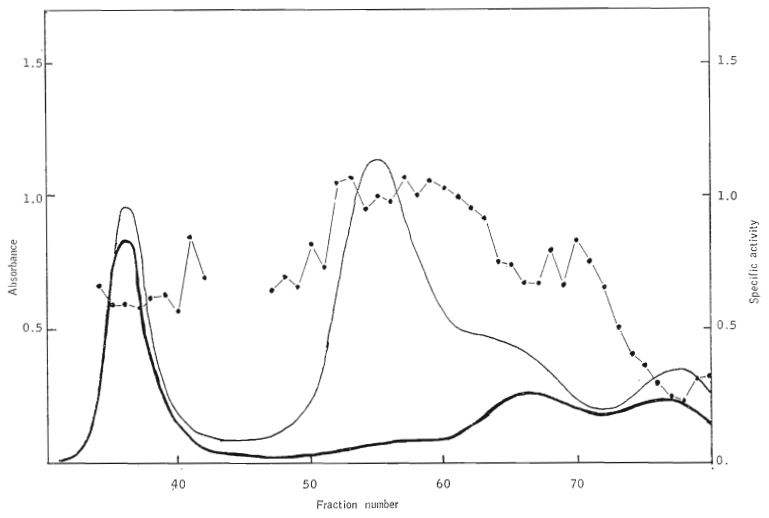

Fig. 9. Gel filtration patterns of the mixture of Hp, Fr. 2 and α-chain. Legends same as in Fig. 5.

complex formed between Hp and native Hb (Table 2).

Fr. 2 which consisted $\alpha\beta$ and β_2 dimers partly reacted with Hp to form a stable complex (Fig. 8), for the increase of complex region was inversely accompanied by the decrease in specific activity (Table 2). Since Hp has no binding site for sole β-chain, it seems to be the $\alpha\beta$ dimer that binds with Hp from Fr. 2 and β_2 dimer would remain unbound (Figs. 6, 7). And this $\alpha\beta$ dimer is considered to be α^1-β^1 dimer rather than α^1-β^2 dimer from the observation of Rosemeyer and Huehns[16].

In the presence of α-chain, more Fr. 2 bound with Hp than was expected from the experiment without α-chain, because of such a low specific activity as 0.60 (Table 2).

The decreased specific activity in the experiment with α chain seems to be due to the binding of non-labeled $\alpha\beta$ dimer which would be regenerated from β_2 dimer in Fr. 2 and PCMB-free α-chain. The PCMB-free α-chain would be regenerated from α-chain masked with PCMB, presumably due to the transition of PCMB from α-chain to labeled Hb. Such a speculation could be supported by the observation presented in Figs. 5 to 7 in which the splitting of the peak corresponding to the labeled Hb (fraction number around 55) into dimer region (fraction number around 65) was observed.

The generation of β_4 tetramer from β_2 dimer of Fr. 2 is also expected

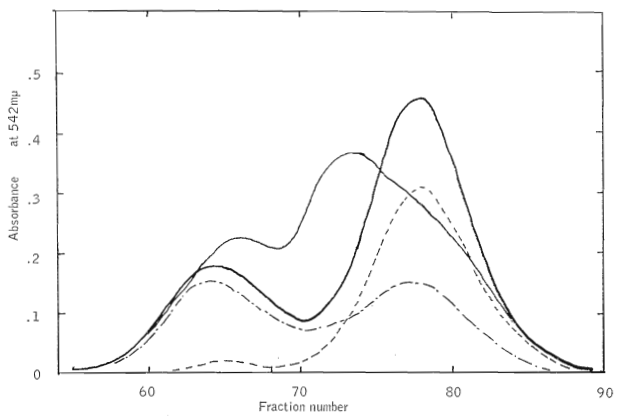

Fig. 10. Gel filtration patterns of α-and β-chains. Absorbance was determined at 542 mμ as HbO_2.
– – – – – – – – : α-chain.
—·—·—·— : First eluate from CM-cellulose column with 0.01M PPB, pH 6.5.
——————— : Synthetic curve of dotted and broken lines.
——————— : Mixture of α-chain and the first eluate from CM-cellulose column with 0.01M PPB, pH 6.5.

Table 2. Protocol for summary of Figs. 5–9.

Sample	Maximal absorbance in Hb-Hp region		Specific activity of Hb-Hp	Maximal absorbance in Hb region		Maximal absorbance in Hb region without Hp
	labeled Hb			labeled Hb		
	without	with		without	with	
Hp+α-chain	260	840	0.95	(m)[1] 270	410	390
Hp+Fr. 1	25	900	0.95	(m) 360	330	310
Hp+Fr. 1+α-chain	980	970	0.05	(m) 330	80	750
Hp+Fr. 2	150	960	0.80	(d)[2] 200	280	300
Hp+Fr. 2+α-chain	600	950	0.60	(d) 210	310	310
				(m) 200	340	380
Hp+β_4	0	850	0.95			
Hp+Hb A_1	850	—	—			
Hp+labeled Hb	—	830	1.00			

Maximal absorbance in each of the Hb-Hp complex and monomer and dimer Hb region were read in the gel filtration pattern. Absorbance of HbO_2 in the experiment without labeled Hb was converted to that of cyanmentHb by calculation. Used value for millimolar extinction of HbO_2 and cyanmentHb was 132 at 414 mμ and 117 at 421 mμ, respectively. As a control, maximal absorbance in Hb region without Hp was indicated in the Table from the value in the different run. As another control, the value from the experiment with β_4, Hb A_1, and labeled Hb was indicated in the lower part of the Table.

1 (m) Monomer region of gel filtration (fraction number around 77)

2 (d) Dimer region of gel filtration (fraction number around 67)

(Fig. 9) because of the lower specific activity of the fraction around number 50, which corresponds to the elution number of β_4 tetramer from another run through Sephadex G-100 column.

Discussion

Alfsen[13] mentioned that β-chain of Hb does not bind with Hp as a part of an $\alpha\beta$ dimer, but a separate site, from the stand point of view that there is little tendency for free α- and β-chain to associate.

In spite of this augument, α- and β-chains seemed to have tendency to associate even when masked with PCMB as has been shown in the experiment of gel filtration; that is, the mixed solution of α- and β-chain is filtrated faster than α-chain and β-chain separately through Sephadex G-100 column (Fig. 10). α-Chain reacts with Hp only when α-chain is present and forms a stable complex (Figs. 6, 7). It is possible that β-chain binds directly with Hp near the site for α-chain and that β-chain interacts with α-chain even when PCMB molecules are present at α-β contact.

In the present experimental condition, Hp was not saturated with α-chain in spite of the presence of excess α-chain. This evidence seems to

suggest that four sites of Hp[13] are not equivalent in respect to the binding ability.

The experiment with Fr. 2, a mixture of $\alpha\beta$ and β, indicated that $\alpha\beta$ dimer masked with PCMB, probably α^1-β^1 or α^2-β^2 dimer[16], binds with Hp with enough stability. From these experiments, the unit dimer of native Hb which binds with Hp is considered to be α^1-β^1 dimer rather than α^1-β^2 dimer.

Acknowledgement

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References

1) Jayle, M. F., and Moretti, J.: Haptoglobin: Biochemical, genetic and physiopathologic aspects. Progr. Hematol., 3: 342–359, 1962.
2) Laurell, C. B.: Purification and properties of different haptoglobins. Clin. Chim. Acta, 4: 79–81, 1959.
3) Shim, B.-S. et al.: Immunological and biochemical investigations of human serum haptoglobin: Composition of haptoglobin-haemoglobin intermediate, haemoglobin binding sites and presence of additional alleles for β-chain. Nature, 207: 1264–1267, 1965.
4) Hamaguchi, H.: Control of haptoglobin metabolism. IV. Physicochemical properties of the hemoglobin-haptoglobin "subunit" and the hemoglobin-haptoglobin complex of human haptoglobin 1-1 type. Proc. Japan Acad., 43: 562–565, 1967.
5) Bunn, H. F.: Effect of sulfhydryl reagents on the binding of human hemoglobin to haptoglobin. J. Lab. Clin. Med., 70: 606–618, 1967.
6) Bunn, H. F.: Subunits dissociation of certain abnormal human hemoglobins. J. Clin. Invest., 48: 126–138, 1969.
7) Chiancone, E. et al.: Studies on the reaction of haptoglobin with haemoglobin and haemoglobin chains. J. Mol. Biol., 34: 347–356, 1968.
8) Nagel, R. L., and Gibson, Q. H.: Kinetics and mechanism of complex formation between hemoglobin and haptoglobin. J. Biol. Chem., 242: 3428–3439, 1967.
9) Peacock, A. C. et al.: Haptoglobin-hemoglobin interaction. Stoichiometry. Biochemistry, 9: 2275–2279, 1970.
10) Nagel, R. L. et al.: Comparative haptoglobin binding properties of oxyhemoglobin and deoxyhemoglobin. J. Biol. Chem., 240: PC 4543–4545, 1965.
11) Chiancone, E. et al.: The combination of haptoglobin 2-2 with the oxy and deoxy forms of human haemoglobin before and after digestion by carboxypeptidase A, and with isolated α-chains. Biochim. Biophys. Acta, 117: 379–386, 1966.
12) Antonini, E. et al.: Studies on carboxypeptidase digests of human hemoglobin. J. Biol. Chem., 236: PC 60–63, 1961.
13) Alfsen, A. et al.: Studies on the reaction of haptoglobin with hemoglobin and hemoglobin chains. II. Kinetics of complex formation. Biochim. Biophys. Acta, 200: 76–80, 1970.
14) Guidotti, G.: Studies on the chemistry of hemoglobin. I. The reactive sulfhydryl groups. J. Biol. Chem., 242: 3673–3684, 1967.

15) Perutz, M. F.: Structure and function of haemoglobin. I. A tentative atomic model of horse oxyhaemoglobin. J. Mol. Biol., 13: 646–668, 1965.
16) Rosemeyer, M. A., and Huehns, E. R.: On the mechanism of the dissociation of haemoglobin. J. Mol. Biol., 25: 253–273, 1967.
17) Park, C. M.: The dimerization of deoxyhemoglobin and of oxyhemoglobin: Evidence for cleavage along the same plane. J. Biol. Chem., 245: 5390–5394, 1970.
18) Briehl, R. W., and Hobbs, J. F.: Ultraviolet difference spectra in human hemoglobin. J. Biol. Chem., 245: 544–554, 1970.
19) Geraci, G. et al.: Preparation and properties of α- and β-chains from human hemoglobin. J. Biol. Chem., 244: 4664–4667, 1969.
20) Boyer, P. D.: Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials. J. Amer. Chem. Soc., 76: 4331–4337, 1954.
21) Laurent, T. C. and Killander, J.: A theory of gel filtration and its experimental verification. J. Chromatogr., 14: 317–330, 1964.
22) Smithies, O.: Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. Biochem. J., 61: 629–641, 1955.
23) Hamaguchi, H.: Purification and some properties of the three common genetic types of haptoglobins and the hemoglobin-haptoglobin complexes. Amer. J. Hum. Genet., 21: 440–456, 1969.
24) Connell, G. E., and Smithies, O.: Human haptoglobins: Estimation and purification. Biochem. J., 72: 115–121, 1959.
25) Vinograd, J., and Hutchinson, W. D.: Carbon-14 labeled hybrids of haemoglobin. Nature, 187: 216–218, 1960.
26) Tyuma, I. et al.: The preparation and properties of the isolated α and β subunits of hemoglobin A. Biochemistry, 5: 2957–2962, 1966.