ウナギのハプトグロビンに関する研究III

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Studies on the Haptoglobin of Eel—III Binding Behavior of Haptoglobin with Hemoglobin (1)

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The binding behavior of eel haptoglobin (Hp) with hemoglobins (Hb's) from the eel and some other sources was examined. Eel Hp combined stoichiometrically with component F, but hardly at all with component S of eel Hb. The amount of component F bound by eel Hp seemed to depend on the aging of the former: *i.e.*, the Hp combined equimolarly with the freshly prepared component F, but combined more than 4 moles per mole with the aged one. Eel Hp did not combine stoichiometrically with the Hb's of several other fishes such as carp, skipjack, loach, *etc.* However, the binding of this Hp with rabbit and dolphin Hb's was stoichiometric and equimolar. On the other hand, rabbit Hp did not combine stoichiometrically with any of the fish Hb's.

In the previous papers,[1,2] we reported the purification and some physico-chemical properties of eel haptoglobin (Hp). In the mammals, the Hp of a given species can combine stoichiometrically and equimolarly with Hb of the same species as well as other species.[3] In contrast, AIZAWA[4] found, by use of the partially purified eel Hp, that it combined stoichiometrically with component F of eel Hb roughly at a molar ratio of 1:2. This phenomenon seemed interesting from the viewpoint of comparative biochemistry and to deserve further examination.

This paper deals with the binding behavior of the highly purified eel Hp with several piscine and mammalian Hb's.

Experimental

Haptoglobins The Hp's of the eel *Anguilla japonica* and the rabbit for reference were isolated and purified as described in the previous paper.[1] Each Hp was concentrated to *ca.* 1% by ultrafiltration and assayed for the binding behavior with Hb as given below. The concentration of Hp was determined by u.v. spectrophotometry.[2]

Hemoglobins The blood was collected by heart puncture or by cutting the head from the eel *A. japonica*, carp *Cyprinus carpio*, plaice *Kareius bicoloratus*, skipjack *Katsuwonus pelamis*, loach *Misgurnus anguillicaudatus*, man-eater shark *Isurus glaucus*, blue-white dolphin *Stenella caeruleo-alba*, and rabbit. Heparin was used as the anticoagulant. The blood cells were separated by centrifugation and hemolyzed as

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usual. The hemolyzate thus obtained was treated with trace amounts each of ferricyanide and cyanide and was used as cyanmet Hb solution. The Hb's of eel, loach, and blue-white dolphin, were separated into components F and S by starch block electrophoresis[1] and used. The concentration of Hb was measured spectrophotometrically at 540 nm in the cyanmet form using its molar extinction coefficient, 11,500.[5]

Binding test of Hp with Hb The solutions of Hp and cyanmetHb of known concentrations were mixed at different proportions at the room temperature. The pH values of the reaction mixtures thus provided were 7–8. The mixtures were then subjected to 3 hr-starch gel electrophoresis at pH 8.4 as described previously.[1] The free Hp and Hb were also run. After completion of the electrophoresis, each gel was sliced horizontally into two halves, one being stained with benzidine and the other with amido black 10B. Bands of Hp–Hb complexes, the free Hp, and the free Hb's, if any, in a given electropherogram were identified as usual. Based on all of those results, a conclusion on the binding behavior of eel or rabbit Hp with a given Hb was drawn. In the case where the Hp–Hb complex and free Hb bands seemed to overlap each other, the metHb was used instead of the cyanmet form.

Results and Discussion

In some preliminary experiments, effects of various factors on the binding behavior of eel Hp with component F of eel Hb were examined. The results showed that the pH (4–9) and concentration (0–0.5 M) of buffer solutions (mostly phosphate and partly veronal) used, the time (0–30 min) and temperature (0–37 °C) of reaction, and the

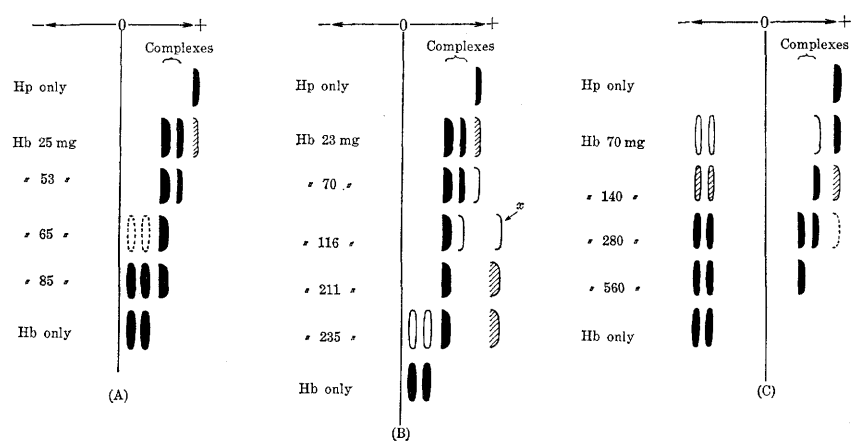

Fig. 1. Binding behavior of eel Hp with its Hb components. (A): freshly prepared component F, (B): one-month aged component F, and (C): freshly prepared component S. The Hb amounts indicated represent those of Hb added per 100 mg of Hp. Complex: Hp–Hb complex. Amido black stain.

derived form of Hb, all do not affect significantly the binding behavior of Hp and Hb, in agreement with the observation with both proteins of mammal.[3] Hence, the test method described above was adopted.

Binding behavior of eel Hp with various Hb's *Components F and S of eel* Different results were obtained depending on the aging of Hb. When the freshly prepared Hb was used, the reaction proceeded stoichiometrically, and two Hp–Hb complex bands appeared at a level of 25 mg Hb/100 mg Hp as shown in Fig. 1A. Small amounts of free Hp was also detected. At a level of 65 mg Hb/100 mg Hp, the free Hp and the fast migrating Hp–Hb complex band completely disappeared and two faint bands of free Hb appeared, thus indicating that the Hp was saturated with Hb just below this level. Taking into account the assumed molecular weights of eel Hp (95,000)[2] and Hb (65,000)*, a molar ratio of 1:1 was calculated for the binding of eel Hp and component F. The complex band which disappeared at 65 mgHb/100 mg Hp could be the half-saturated complex consisting of one molecule of Hp and half molecule of Hb.[6]

When component F was stored at 2–4°C for one month in the cyanmet form and used, both free Hb bands did not appear until a level of 235 mg Hb/100 mg Hp was reached, as Fig. 1B shows. Incidentally, a diffuse band designated "x" which stained weakly with benzidine and amido black 10B appeared above a level of 116 mg Hb/100 mg Hp. The nature of this diffuse band will be reported in the succeeding paper. Eel Hp was saturated near a level of 235 mg Hb/100 mg Hp as described above. This means that the molar ratio of Hp and Hb was about 1:3, suggesting that there is a positive relationship between the storage time of component F and its amount bound by eel Hp.

Then, the freshly prepared component F was stored at 2–4°C in the oxy form and

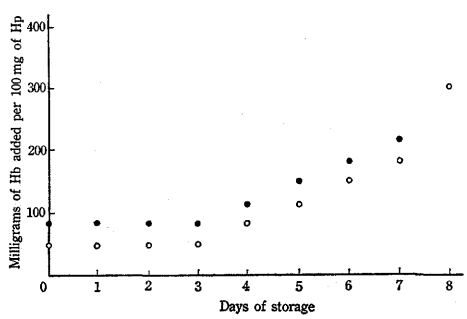

Fig. 2. Influence of Hb aging on the binding behavior of eel Hp with component F. Hb used was stored in the oxy form at 4°C and converted into cyanmet form when tested. ●: The level of Hb above which the Hp was surely saturated. ○: The level of Hb under which the Hp was not saturated as yet.

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examined for changes in its amount bound by eel Hp, after conversion into the cyanmet form. As shown in Fig. 2, the Hb was bound equimolarly by eel Hp until the third day. After the 4th day, however, the amount of Hb bound was increased gradually. It was approximately 2 moles/mole of Hp on the 6th day, just coinciding with the data of AIZAWA.[4] When 8-day old Hb was used, any free Hb band did not appear at a level of 300 mg Hb/100 mg Hp. This means that one molecule of Hp combined with more than 4 molecules of Hb. Curiously enough, however, the electrophoretic mobility of the Hp–Hb complexes thus formed did not differ apparently from that of the equimolar complex.

In the present experiments was used the eel Hp specimen which was prepared under special care as to both the time of its contact with NaCl and the time of its adsorption onto DEAE-cellulose as described previously.[1] In contrast, the eel Hp specimen purified by the method of CONNELL *et al.*[7] combined with component F stoichiometrically and equimolarly, irrespective of the aging of the latter.

On the other hand, both free Hp and free component S bands appeared in addition to Hp–Hb complex bands at Hb levels of 70–280 mg/100 mg (Fig. 1C). In other words, their binding was not stoichiometric. The results were regardless of the

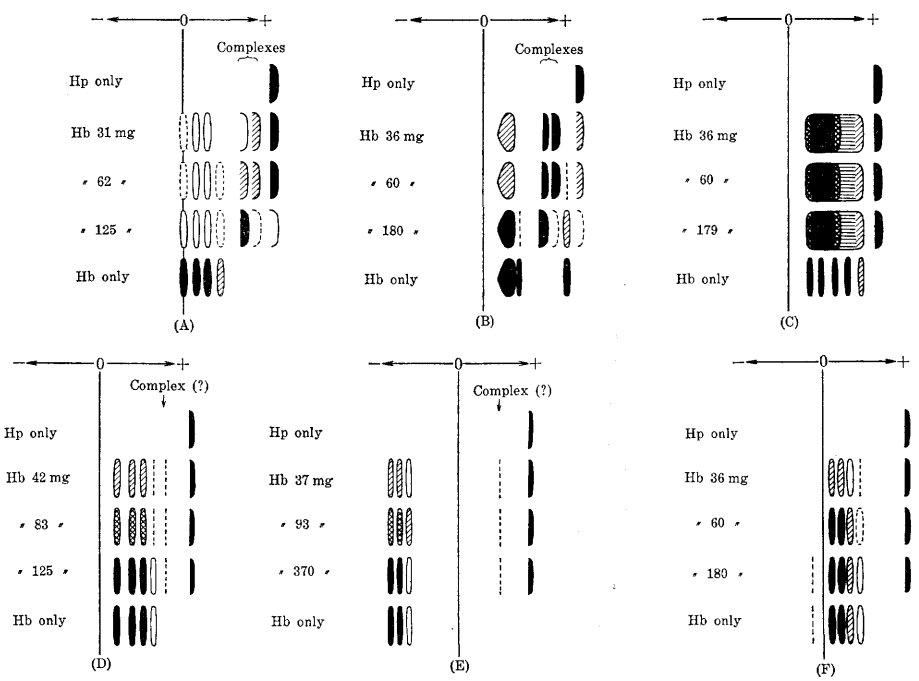

Fig. 3. Binding behavior of eel Hp with various piscine Hb's. (A): carp Hb, (B): skipjack Hb, (C): plaice Hb, (D): component F of loach Hb, (E): component S of loach Hb, and (F): man-eater shark Hb. Refer to the legend in Fig. 1. Amido black stain.

againg of the Hb specimen. Any unidentified band was not observed with this component.

Other piscine Hb's Only freshly prepared specimens of Hb's were used. As illustrated in Fig. 3A, large to appreciable amount of eel Hp and carp Hb remained unreacted at Hb levels of 31–125 mg/100 mg Hp, indicating that their binding reaction did not proceed stoichiometrically. Almost the same results were obtained when skipjack Hb was used instead of carp Hb (Fig. 3B).

The binding behavior of eel Hp with plaice Hb is shown in Fig. 3C. The free Hp band remained apparently unreacted irrespective of the amount of Hb added. It was also noted that the originally well-defined bands of plaice Hb became obscure in the electropherograms of its mixtures with eel Hp. This phenomenon may suggest that eel Hp has some denaturing action on this Hb.

As seen in Figs. 3D and 3E, eel Hp combined hardly with both Hb components of loach. Only a faint band, possibly Hp–Hb complex, was detected in either case. Comparable results were also obtained in the combination of eel Hp with man-eater shark Hb (Fig. 3F).

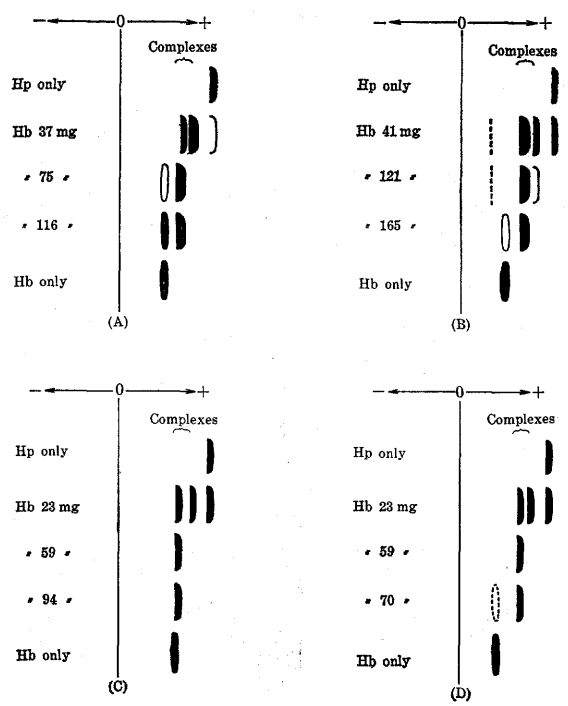

Fig. 4. Binding behavior of eel Hp with some mammalian Hb's. (A): freshly prepared rabbit Hb, (B): one-month aged rabbit Hb, (C): freshly prepared component F of dolphin, and (D): freshly prepared component S of dolphin. Refer to the legend in Fig. 1. Amido black stain.

Mammalian Hb's As given in Fig. 4A, the freshly prepared rabbit Hb combined stoichiometrically with eel Hp. Eel Hp was saturated with the Hb just below a level of 75 mg/100 mg Hp. The combination of eel Hp with rabbit Hb (assumed molecular weight, 67,000[8]) was found to be equimolar.

The results of binding test of eel Hp with the rabbit Hb specimen which was stored at 2–4°C for a month in the oxy form are shown in Fig. 4B. In this case, the reaction appeared to proceed stoichiometrically and the free Hb band was not observed until the Hb level was elevated up to 165 mg/100 mg Hp. Hence, the binding ratio of Hp and Hb was roughly calculated to be 1 : 2 on the molar basis. In the case of component F of eel, the same binding ratio was attained by a 4-day storage. This difference could be due to that in their stability. Incidentally, a faint benzidine-positive band, "x", appeared between the Hb band and the origin. This may correspond to the unidentified diffuse band which was observed in the binding test with the aged component F of eel Hb.

The freshly prepared component F of blue-white dolphin Hb seemed to bind stoichiometrically with eel Hp as shown in Fig. 4C. The binding ratio was not calculated since the Hp–Hb complex and free Hb bands practically overlapped each other. Component S of dolphin bound stoichiometrically with eel Hp. As seen in Fig. 4D, eel Hp was saturated with the Hb near a Hb level of 70 mg/100 mg Hp, suggesting that the binding was equimolar.

Binding behavior of rabbit Hp with various Hb's *Components F and S of eel* When the mixturers of rabbit Hp and the freshly prepared component F were electrophoresed, there appeared a free Hp band and two free Hb bands in addition to the Hp–Hb complex band at almost all the Hb levels, thus indicating that the reaction did not proceed stoichiometrically (Fig. 5A). Besides, a faint unidentified band, "x", appeared irrespective of the level of Hb. The band was weakly benzindine-positive.

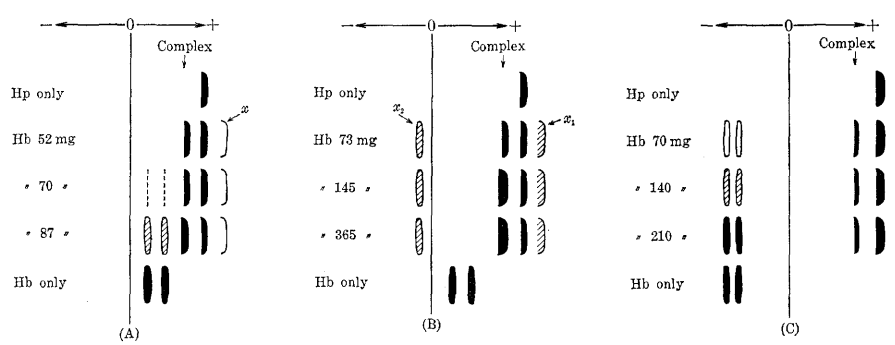

Fig. 5. Binding behavior of rabbit Hp with eel Hb components. (A): freshly prepared component F, (B): one-week aged component F, and (C): freshly prepared component S. Refer to the legend in Fig. 1. Amido black stain.

In Fig. 5B is shown the results with the component F which was kept at 2–4°C for a week in the oxy form. At a Hb level of 73 mg/100 mg Hp, a Hp–Hb complex band appeared with the free rabbit Hp band and two unidentified diffuse bands, x_1 and x_2, one in the anodic and the other in the cathodic side. Essentially the same patterns were observed at higher Hb levels up to 365 mg/100 mg Hp. No free Hb bands appeared throughout. Incidentally, the same results as above were obtained when the rabbit Hp specimen-prepared by CONNELL's method[7] was used instead. This fact suggests that rabbit Hp is more stable than eel Hp.

Component S did not combine stoichiometrically with rabbit Hp either, as shown in Fig. 5C.

Other piscine Hb's As illustrated in Fig. 6A, the binding of rabbit Hp with carp Hb was not stoichiometric. So was the case with skipjack Hb (Fig. 6B).

The results of binding test of rabbit Hp with plaice Hb are given in Fig. 6C. At the three Hb levels tested, no Hp–Hb complex band appeared. Instead, the rabbit Hp band remained unchanged throughout, and in addition, a diffuse broad band roughly covering the area where plaice Hb bands were located otherwise, was observed.

As shown in Figs. 6D, 6E and 6F, rabbit Hp did not bind with both components of loach, and with man-eater shark Hb either.

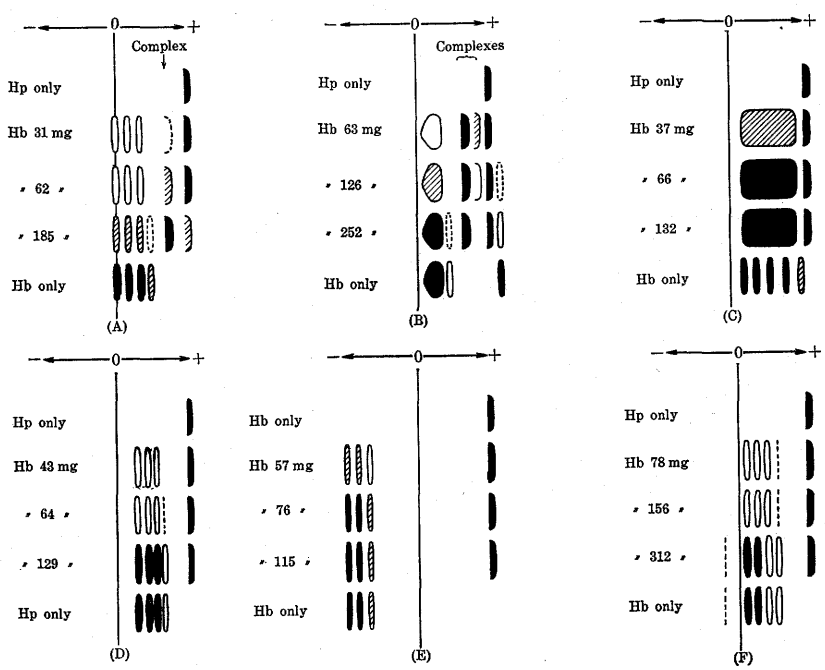

Fig. 6. Binding behavior of rabbit Hp with freshly prepared piscine Hb's. (A): carp Hb, (B): skipjack Hb, (C): plaice Hb, (D): component F of loach Hb, (E): component S of loach Hb, and (F): man-eater shark Hb. Refer to the legend in Fig. 1. Amido black stain.

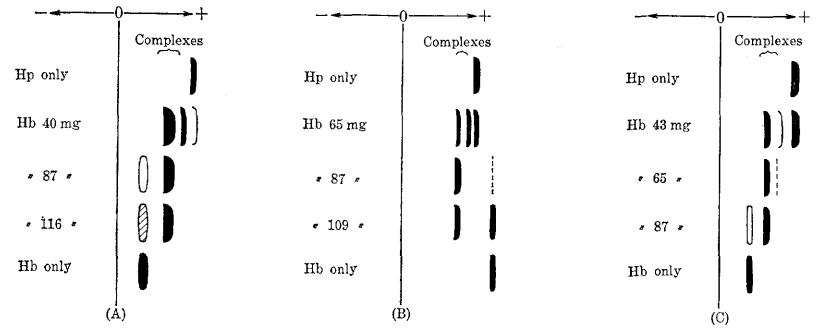

Fig. 7. Binding behavior of rabbit Hp with freshly prepared mammalian Hb's. (A): rabbit Hb, (B): component F of dolphin, and (C): component S of dolphin. Refer to the legend in Fig. 1. Amido black stain.

Mammalian Hb's In the case of rabbit Hb, the cyanmet form was found unsuitable, because it appeared overlapping with the Hp–Hb complex in the electropherogram. The binding test was, therefore, carried out in the met form. As seen in Fig. 7A, rabbit Hp combined stoichiometrically with its Hb. A faint band of free Hb appeared at its level of 87 mg/100 mg Hp. Since the molecular weights of rabbit Hp and Hb are approximately 70,000[5] and 67,000[8] respectively, these results mean that their binding proceeded equimolarly, as has already been reported.[9] The results were essentially the same when rabbit Hb was aged for 2 weeks at 2–4°C in the oxy form and used. Similarly, the binding of rabbit Hp with both Hb components of blue-white dolphin was also stoichiometric and equimolar, as seen is Figs. 7B and 7C.

As described above, eel Hp clearly differs from rabbit Hp in the binding behavior with component F of eel Hb: Eel Hp bound stoichiometrically with it, while rabbit Hp did not. This seems to be manifestation of their specific specificities.

In the combination with the other piscine and mammalian Hb's, eel and rabbit Hp's resemble each other: Both Hp's bound hardly with component S of eel, and with Hb's from carp, skipjack, loach, and so on. This is in a striking contrast to the binding of Hb and Hb in the mammal, the binding which proceeds stoichiometrically in any pair of both proteins.[3]

In the binding with plaice Hb, on the other hand, both Hp's behaved similarly again. Instead of the free Hb bands, a diffuse broad benzidine-positive band appeared in the electropherogram, irrespective of the level of Hb added. This phenomenon, together with the nature of some unidentified benzidine-positive bands, which were rather often encountered in the present study, will be dealt with in the succeeding paper.

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