IMMUNOLOGICAL STUDIES IN THE MULTIPLE HEMOGLOBINS OF TADPOLE AND FROG OF *RANA CATESBEIANA*

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1. Introduction

It has long been known that the hemoglobins of tadpole and frog of *Rana catesbeiana* occur in multiple forms and the major components of both hemoglobins are remarkably different in function and structure from one another [1–7], whereas little information is available on the properties of their minor components. In an attempt to clarify the immunological properties of multiple hemoglobins in the tadpole and the frog, we have found that all of the hemoglobin components in the frog, as well as in the tadpole, are immunologically indistinguishable from each other. No immunological cross-reactivity was detected between the components of tadpole and frog hemoglobins. Formation of two precipitin bands between the tadpole hemoglobin or globin and rabbit anti-tadpole hemoglobin serum is also discussed from the point of the dissociation–association equilibrium of the protein.

2. Materials and methods

Tadpoles and adult bullfrogs (*Rana catesbeianal*) were purchased from the Shiihashi Biological Supply, Tokyo. The hemolysates were prepared as described previously [3]. Tadpole and frog hemoglobins were each fractionated into four fractions (named T1-T4 for the tadpole and F1-F4 for the frog) by carboxymethyl (CM)-cellulose column chromatography with a linear pH gradient, following the method of Aggarwal and Riggs [5]. As may be seen in fig. 1A, each of three fractions (T1-T3) was resolved into several bands by polyacrylamide gel electrophoresis. Electrophoresis of each of the main bands was repeated at least three times successively by cutting off the main band and layering it directly on the top of the next disc until the band showed single components (fig. 1B). The main bands were eluted from the gel with 0.15 M NaCl solution. Other hemoglobin fractions T4, F1, F2, F3 and F4 were also purified in the same way. These highly purified hemoglobin components were named as T′1–T′4 for tadpole and F′1–F′4 for frog. Analytical polyacrylamide gel electrophoresis was carried out following the method of Davis [8]. Rabbit anti-hemoglobin sera (anti-T2 and anti-F3) were prepared by injecting the major tadpole hemoglobin (T2) or the frog hemoglobin (F3) into the rabbits respectively. Two dimensional immunodiffusion in agar was carried out by Ouchterlony's method [9], using electrophoretically purified hemoglobin components unless otherwise noted. Globin were prepared by the acid–acetone method [10]. Sedimentation analysis was performed with a Hitachi model UCA-1A ultracentrifuge.

3. Results and discussion

As shown in fig. 2A, two precipitin bands developed between anti-T2 and T′2–T′4 components of tadpole

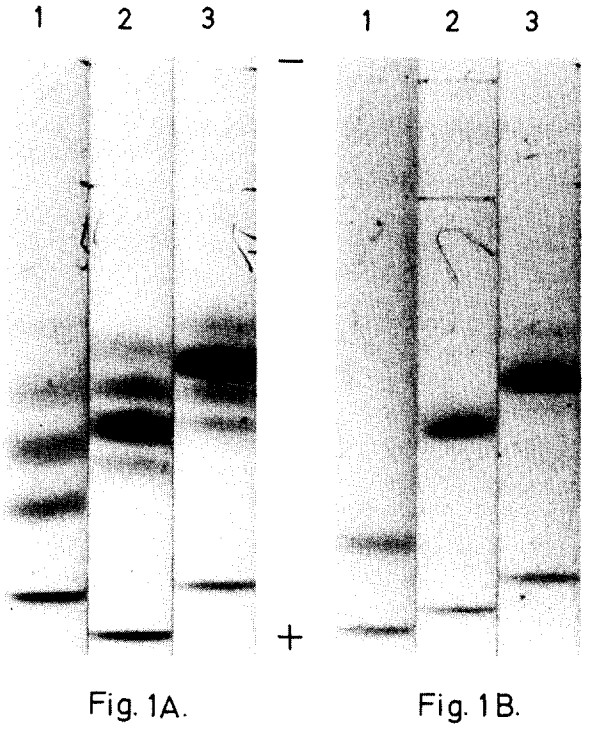

Fig. 1. Polyacrylamide discontinuous gel electrophoresis of tadpole hemoglobin. A) Electrophoretic pattern of tadpole hemoglobin fractions prepared by CM-cellulose column chromatography. Gel 1, 2 and 3 contained fractions T1, T2 and T3 respectively; B) Pattern after the third electrophoresis following CM-cellulose column chromatography. Gel 1, 2 and 3 contained the main band from T1, T2 and T3 respectively.

hemoglobin and complete fusions were observed between the components. The outer band stained well with benzidine dye, whereas the inner bands stained less intensely. This is in agreement with the result of Wise [6] which was obtained by immunoelectrophoresis, while Maniatis and Ingram [7] mentioned that the inner band was incapable of staining. It should be noted that the T′1 component, containing a hemoglobin concentration corresponding to T′2–T′4, developed only a single precipitin band which fused with the inner band of the T′2–T′4 components. From these results it appears that the components T′2–T′4, were immunologically indistinguishable from each other, and that the formation of the two precipiting bands was not due to contamination of either one of the different hemoglobin components or of the other protein. Since Aggarwal and Riggs [5] found considerable variability in amino acid composition of the hemoglobin components in tadpole, as well as in frog, it is probable that there exist genetically different hemoglobins in the hemolysate, which were immunologically indistinguishable. Considering these facts, it is most likely that each component, despite differences of primary sequence, has a quite similar immunological determinand in its structure. Using single amino acid mutants of human hemoglobin, Reichlin [11] pointed out very recently that mutations antigenically indistinguishable from normal adult hemoglobin are all located in the region of the primary sequence where rabbit and human hemoglobin are identical. On the basis of his proposal it is not surprising that the hemoglobin components of tadpole are immunologically indistinguishable from each other, even if they have marked differences in primary structure.

Maniatis and Ingram [7] reported that two precipitin bands developed between the major component of tadpole hemoglobin and its antiserum in the Ouchterlony technique, while tadpole globin formed only one precipitin line which merged completely with the inner band between hemoglobin and the same antiserum. From these facts, they proposed that the outer band might be due to hemoglobin and the inner one to globin. In contrast to their results, tadpole globin formed two precipitin bands which fused completely with the corresponding precipitin bands developed by hemoglobin (as shown in fig. 2B). Of these, the outer band disappeared with decreasing concentration of the globin. A similar result was observed with the T′2 component. As is evident from fig. 2C, T′2 hemoglobin at lower concentration showed only the inner band. Therefore, protein concentration seems to play a role in two bands formation with either globin or hemoglobin. That the development of the outer precipitin band might be due to contamination by hemoglobin of the globin solution can be excluded by the following fact. The globin solution of 60 μg/ml, the lowest concentration with which two precipitin bands were developed, contained less than 0.6 μg/ml hemoglobin. At this concentration, the hemoglobin never developed any precipitin band under the present experimental conditions. As mentioned above, T′1 formed only the inner band (fig. 2A). Aggarwal and Riggs [5] reported that a fraction of the tadpole hemoglobin component eluted early from the CM-cellulose column, correspond-

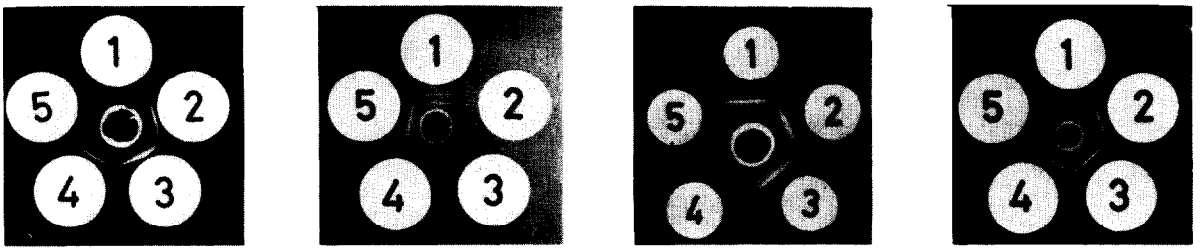

Fig.2A. Fig.2B. Fig.2C. Fig.2D.

Fig. 2. Two-dimensional immunodiffusion (unstained). Each well contained 2 μl of the protein solution: A) Center well, anti-T2; well 1, T'1; well 2, T'2; well 3, T'3; well 4, T'4; well 5, saline; B) Center well, anti-T2; well 1, T'2 (3 mg/ml); well 2, tadpole globin (30 μg/ml); well 3, same as well 1; well 4, tadpole globin (60 μg/ml); well 5, tadpole globin (2 mg/ml); C) Center well, anti-T2; well 1, T'2 (1 mg/ml); well 2, T'2 (0.5 mg/ml); well 3, T'2 (0.25 mg/ml); well 4, T'2 (0.125 mg/ml); well 5, T'2 (0.0625 mg/ml); D) Center well, anti-F3; well 1, F'1; well 2, F'2; well 3, F'3; well 4, F'4; well 5, same as well 1.

ing to our T'1 component, would not be a tetrameric form but a dimeric form of hemoglobin. Generally, it is known that liganded hemoglobin dissociates from $\alpha_2\beta_2$ tetramer into $\alpha\beta$ dimer symmetrically at lower concentration below 10^{-6} M [12]. This tendency to splitting is especially remarkable in globin. Human globin has been shown to have a sedimentation coefficient of 2.5 S and mean molecular weight of 42 000 [10] even at a concentration as high as 1%. This fact has been interpreted as being due to the co-existence of dimers and tetramers of the globin in a equilibrium state. In the present experiments, the sedimentation coefficient (S^0_{20w}) of the tadpole globin was estimated to be 2.3 S at pH 7.0 in 0.01 M phosphate buffer by ultracentrifugal analysis, suggesting that tadpole globin is also in dimer–tetramer equilibrium. All these results suggest the possibility that the inner and outer bands developed by the hemoglobin and globin may result from the dimer and tetramer, respectively. Since it has been shown that when human hemoglobin is dissociated into monomer polypeptide chains, new antigenic sites are exposed which are not present in the native tetramer [13], it would be very probable that dissociation of tadpole hemoglobin into dimers may also change the immunological determinant.

F'3 component of frog hemoglobin developed only one precipitin band against anti-F3 at any concentration so far examined on gel diffusion analysis as shown in fig. 2D, and complete fusion of the precipitin band was observed between frog hemoglobin components. It is interesting that the frog hemoglobin components are immunologically indistinguishable from one another, despite quite different electrophoretic mobilities, as observed with tadpole hemoglobin. Frog globin also formed only one precipitin band against anti-F3 which merged completely with the hemoglobin band, even at a high concentration, differing from the tadpole globin. The sedimentation coefficient of the frog globin was found to be 2.3 S, suggesting co-existence of dimers and tetramers. The reason why the hemoglobin and globin of frog show only one precipitin line in contrast to those of tadpole is not known as yet, however, it is possible to suppose that the frog globin and hemoglobin do not change their immunological determinant on dissociation into smaller subunits.

No cross-reactivity was detected between major components of tadpole hemoglobin (T'2) and frog hemoglobin (F'3) on Ouchterlony plates in agreement with Maniatis and Ingram [7]. In addition, the minor components of tadpole and frog hemoglobins also failed to show any cross-reaction, suggesting that frog and tadpole have no common hemoglobin component so far examined, while they have a component very similar in chromatographic behaviour on CM-cellulose column [3].

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