HEMOGLOBIN HETEROGENEITY IN THE CAT*

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We have reported (1) that hemolysates of cat blood contain two hemoglobins that are of interest because of their relatively low oxygen affinity in comparison with human and other mammalian hemoglobins. The two components, designated major and minor[1] on the basis of their relative concentrations, were considered to be different tetramers. This interpretation was supported by differences in peptide maps of tryptic digests prepared from the isolated proteins. However, Sullivan (2) has been unable to resolve more than one hemoglobin by starch gel electrophoresis and has suggested the possibility that one of our two components was a polymerization product of the other. Polymerization by oxidation and disulfide bond formation between tetrameric molecules was proposed. Such an explanation seemed reasonable since cat hemoglobin contains eight reactive -SH per molecule (3,4) and evidence for polymer formation by disulfide linkage had been obtained with bullfrog and turtle hemoglobins (5). The results presented in this paper, however, show that the two cat hemoglobins contain identical α but different non-α (β) subunits. Furthermore, no evidence could be found for the polymerization of tetramers by disulfide bond formation.

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[1]The major and minor components are now designated cat HbA and cat HbB respectively.

Methods and Procedure

The procedures for collection of blood, preparation of hemoglobin solutions, and the isolation of the two hemoglobins from cat blood have been described previously (1). Hemoglobin concentration was determined by the cyanmethemoglobin method (6).

Spectrophotometric titration of "reactive" -SH groups (7) was carried out by addition of increments of a 2.25 x 10^{-5}M solution of hemoglobin to 2.5 - 3.0 x 10^{-5}M pMB in 0.015 M phosphate buffer pH 7.0, and in a final volume of 5 ml. Increase in absorbance at 255 mμ was read in a Beckman DU spectrophotometer.

Reaction of intact red blood cell (RBC) suspensions with N-ethyl maleimide (NEM) was carried out in isotonic phosphate buffer pH 6.8 for 1 hour (8). The initial reaction mixture contained 2-fold excess of NEM based on a content of eight -SH groups per molecule. Aliquots of the reaction mixture were deproteinized with 10% perchloric acid and NEM uptake was measured in the filtrate at 300 mμ (8). The remainder of the RBC suspension was centrifuged for 5 min at 2500 rpm, the supernatant was removed, and the cells were washed three times with isotonic saline. The packed cells were then hemolyzed with two volumes of distilled water. The hemolysate was centrifuged at 20,000 x g for 30 min to remove the stroma. Aliquots of the clear hemoglobin solutions were then taken for starch gel electrophoresis.

Horizontal starch gel electrophoresis was conducted in a chamber with a gel thickness of about 1.5 mm. Runs were carried out at 4°C in Tris-EDTA-Borate pH 8.4 (9) and the hemoglobins were stained with benzidene or amidoschwarz. The gel included 0.01 M mercaptoethanol for electrophoresis of 1.5 - 5.0 x 10^{-3}M hemoglobin solutions that were previously exposed to 0.01 - 3.5 M mercaptoethanol under a CO atmosphere for periods up to 48 hours.

For the preparation of hybrid hemoglobins, equal volumes of 1 - 2.5% solutions of carbon monoxy derivatives of human HbA and cat HbA or human HbA and cat HbB were mixed and dialyzed at 4°C for about 20 hours against 0.2 M

acetate buffer pH 4.7. The solutions were then dialyzed against several changes of 0.05 M Tris-HCl buffer pH 8.15 over a 24 hour period and analyzed by starch gel electrophoresis.

Results

Table 1 shows the pMB titration data for hemoglobins in human and cat hemolysates and for the isolated cat HbA and cat HbB. The number of -SH in cat methemoglobin prepared by ferricyanide oxidation is also shown. In comparison with the human HbA molecule which combines with two pMB, both cat hemoglobins combine with eight. The same results are obtained even after the hemoglobin solutions have been allowed to stand in the refrigerator for several months or after complete conversion to methemoglobin with ferricyanide.

Table I

pMB Reactive -SH Groups in Human and Cat Hemoglobins

Sample	Moles pMB bound per Mole Hb
Human Hemolysate	2.3
Cat Hemolysate	7.9
Cat HbA	7.6
Cat HbB	8.4
Oxidized Cat Hemolysate (Cat MetHb)	8.2

The effect of mercaptoethanol on the relative amounts of cat HbA and cat HbB is shown in figure 1a. Similar patterns were obtained when the hemoglobins were exposed to 0.01 - 3.5 M mercaptoethanol for 48 hours. The results show that the reducing agent does not cause interconversion of one component to the other.

Figure 1b shows gel electrophoresis patterns of hemoglobins prepared from NEM-treated and untreated erythrocytes. The two components in the untreated sample are also found in the same proportions in the NEM reacted solution. The only notable difference is the slightly increased anodic mobilities of the NEM-

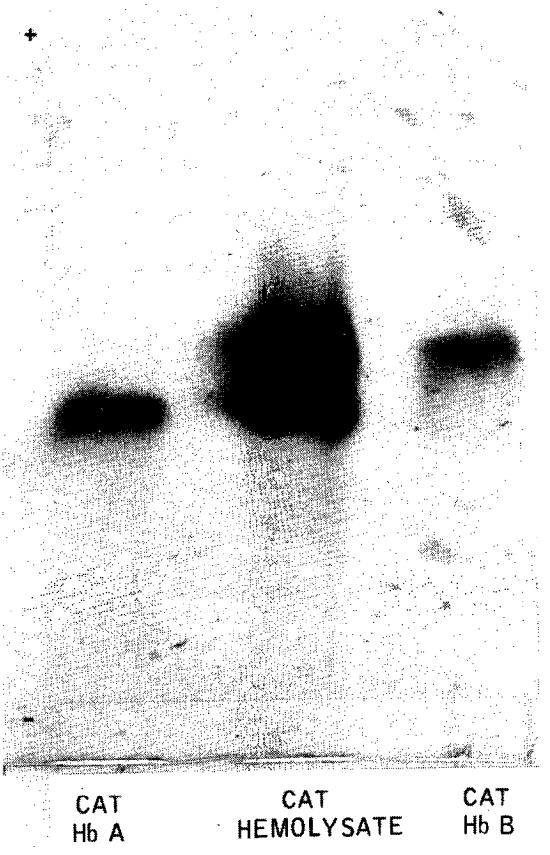

Fig. 1a

Fig. 1b

Fig. 1a. Starch gel electrophoresis of cat hemoglobins in Tris-EDTA-Borate at pH 8.4. The hemoglobin solutions were treated with 0.01M mercaptoethanol before application. The gel buffer also contained 0.01M mercaptoethanol. Stained with amidoschwarz.

Fig. 1b. Starch gel electrophoresis of cat hemoglobins prepared from unreacted (control) and NEM-reacted erythrocytes. Tris-EDTA-Borate, pH 8.4, 15 hrs. Stained with benzidene.

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hemoglobins. Analysis for NEM uptake in this preparation had shown that six to seven equivalents of -SH per molecule of hemoglobin were alkylated.

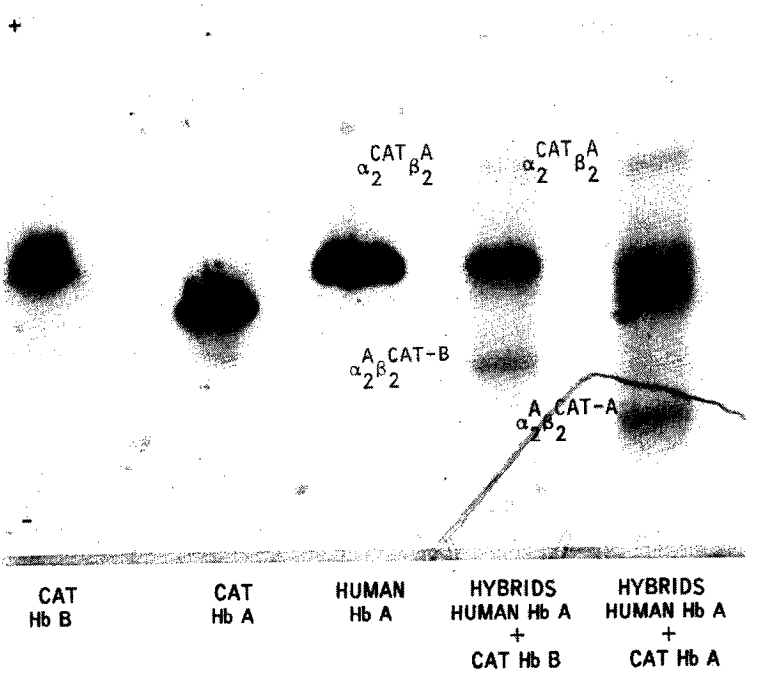

Fig. 2 Starch gel electrophoresis of cat HbA, cat HbB, human HbA, and the human-cat hybridization mixtures. The subunit components of the hybrid molecules are designated. Electrophoresis in Tris-EDTA-Borate pH 8.4.

Figure 2 shows the electrophoretic patterns of dissociation-recombination mixtures of human HbA with both cat HbA and cat HbB. Four components are resolved from the human HbA-cat HbA mixture: the parent hemoglobins and the two hybrids. The fastest and slowest moving hybrids were identified earlier as $\alpha_2^{CAT}\beta_2^{A}$ and $\alpha_2^{A}\beta_2^{CAT}$ respectively (3,4). Only three components are seen in the human HbA-cat HbB mixture since the middle band contains a mixture of human HbA and cat HbB that is not resolved in this system. The two hybrids, however, are readily separated. The same hybrid component exhibiting the most rapid anodic mobility, $\alpha_2^{CAT}\beta_2^{A}$, appears in both hybridization mixtures. On the other hand,

the slow moving hybrid components corresponding to $\alpha_2^A\beta_2^{CAT}$ are clearly different. The results show that the two cat hemoglobins contain identical α and different β chains.

Discussion

The results of pMB titration and of electrophoresis following treatment with mercaptoethanol do not support the suggestion of Sullivan (2) that one of the cat hemoglobins is a polymer derived from the other by formation of disulfide linkages. Both cat hemoglobins contain eight free -SH groups and treatment with mercaptoethanol does not alter their relative concentration. Riggs et al. (5) presented evidence for the formation of a 7S from a 4S hemoglobin component in frog and turtle hemolysates. The 7S component was reconverted to 4S on treatment with mercaptoethanol and, if NEM were added, the 7S component did not form. Our studies on NEM-reacted cat erythrocytes show that, although NEM rapidly reacts with intraerythrocytic thiols (10), the relative amounts of the two hemoglobins remain unaltered.

Preliminary data on Sephadex filtration (11) also rule out polymer formation. Cat HbA and cat HbB elute in the same position and slightly after human hemoglobin from a 1 x 150 cm column of Sephadex G-100. In addition, differences in amino acid composition are found for the two hemoglobins (11).

The formation of identical $\alpha_2^{CAT}\beta_2^A$ but different $\alpha_2^A\beta_2^{CAT}$ hybrid molecules from human HbA-cat HbA and human HbA-cat-HbB mixtures clearly indicates that the two cat hemoglobins differ in the structure of their β-chains. Peptide maps of α^{CAT} and β^{CAT} chains from isolated cat HbA and cat HbB also show that the two proteins contain identical α but different β chains (11). The structures of cat HbA and cat HbB may thus be designated $\alpha_2^{CAT}\beta_2^{CAT\text{-}A}$ and $\alpha_2^{CAT}\beta_2^{CAT\text{-}B}$ respectively

Although we have routinely separated and isolated the two components from cat blood, others (2,12,13) have reported only a single hemoglobin band on electrophoresis. Differences in animals may account for these results. However, the relative resolving power of the electrophoretic systems employed may also account for these differences. It has been reported that cat and human hemoglobin

exhibit the same electrophoretic mobility at pH 8.4 (12,13). However, the major component (CAT HbA) shows a distinctly lower mobility in our thin-gel electrophoresis system at this pH. Only the minor component (Cat HbB), which varies in concentration from a trace to about 50% of the total hemoglobin, shows a mobility nearly identical to that of human HbA. It should be noted that the two cat hemoglobins are readily separated by chromatography on IRC-50, using 0.05M phosphate buffer, pH 6.6-6.7.

No definitive explanation is yet available for the variation in the amount of the minor component found in cats. Although unusual, this is probably the result of alterations in the control of β-chain synthesis. The switch from HbGower to HbF to HbA during early human development, as well as changes in rates of α and β chain synthesis in the thalassemias (14), are examples of such a phenomenon. A further example occurs in sheep where a hemoglobin that is normally present in low concentration (HbC) increases when the animal is made anemic (15,16). The genetic mechanism that may account for variations in the relative proportions of cat HbA and cat HbB is presently under investigation.

Acknowledgments

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