EXCHANGE OF HEME AMONG HEMOGLOBIN MOLECULES*

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Communicated by W. B. Castle, July 11, 1966

Although there have been considerable advances in the understanding of the structural relationship of heme to globin, little is known about the stability of the heme: globin linkage under physiological conditions. With the use of isotopically labeled human hemoglobin, we have observed that intact heme groups transfer readily from one molecule to another.

Methods.—Two easily separable normal human hemoglobins, F and A, were used. Hemoglobin A was prepared from a water hemolysate of washed red cells, and then purified by cation-exchange chromatography by the method of Allen and co-workers.[1] The buffer employed was identical in pH and ionic strength to their "developer #2," but lacked cyanide. In like manner, hemoglobin F was prepared from red cells of normal umbilical cord blood. Fe^{59} hemoglobin F was prepared after incubating reticulocyte-rich cord blood cells with Fe^{59} bound to plasma transferrin. Fe^{59} hemoglobin A was prepared similarly from the red cells of adult patients with elevated reticulocyte levels. In parallel experiments, C^{14}-2-glycine was used for labeling both the porphyrin and globin moieties, and C^{14}-leucine for labeling globin alone. Ferrihemoglobin was prepared by the addition to oxyhemoglobin, at pH 6.8, of 1.2 moles potassium ferricyanide per heme, followed by dialysis against the incubation buffer. Unless stated otherwise, labeled hemoglobin F was incubated with an equal quantity of unlabeled hemoglobin A at 37°C in phosphate buffer, pH 7.18, ionic strength 0.09. It was found that the reaction to be described could be terminated by immersion in ice water or by adding an excess of neutral cyanide. After dialysis in the cold against "developer #2" containing 0.01 *M* cyanide, the hemoglobin mixtures were separated by cation-exchange chromatography.[1] The hemoglobin concentration of each fraction was determined as cyanmethemoglobin. Fe^{59} activity of each fraction was measured with a well-type scintillation counter. C^{14} activity was measured with a "low-background" gas flow counter on planchet-dried aliquots of hemoglobin, heme,[2] and globin.[3] The heme and globin were isolated from each fraction after the addition of unlabeled cyanmethemoglobin as "carrier." Suitable corrections for recovery and self-absorption were made. The specific activity was calculated from the activity and concentration of each fraction, and was expressed as cpm/mg of hemoglobin. A weighted mean of the specific activities of neighboring hemoglobin-rich fractions was calculated. If hemes exchanged freely among hemoglobin molecules, then at equilibrium, the specific activity of the newly labeled hemoglobin would be 50 per cent of the initial specific activity of the originally labeled hemoglobin. The quotient of the specific activity of the newly labeled hemoglobin, at time t over one half the specific activity of the originally labeled hemoglobin, at time 0, is a measure of the per cent of heme exchange. Thus, if hemoglobin F labeled in the hemes is incubated with an equal amount of unlabeled hemoglobin A, the per cent heme exchange = 2 spec. act. A_t/spec. act. F_0.

Subunit hybrids were prepared by a modification of the method of Huehns.[4] Mixtures of equal amounts of carboxyhemoglobin A and of carboxyhemoglobin F, in which only one or the other of the two hemoglobins was labeled with Fe^{59}, were dialyzed vs. 0.1 *M* acetate buffer, pH 4.5, for 48 hr at 4°C. The hemoglobin mixture was then dialyzed against the incubation buffer, after which the F and A hemoglobins were separated in the usual manner. Hemoglobin F labeled only in the alpha chains was recovered, according to the following reaction:

$$\alpha_2{}^*\beta_2{}^* + \alpha_2\gamma_2 \rightleftharpoons \alpha_2{}^*\gamma_2 + \alpha_2\beta_2{}^*.$$

In parallel manner, hemoglobin A labeled only in its alpha chain was prepared. Yields of selectively labeled hemoglobin were generally 50 per cent of theoretical.

Results.—When Fe^{59}-labeled ferrihemoglobin F was incubated at 37°C, pH 7.2, for 16 hr with unlabeled ferrihemoglobin A, the specific activities of the two hemoglobins were found to have become nearly equivalent (Table 1). In contrast, when ferrihemoglobin F was labeled only in the globin with C^{14}-leucine, only a negligible amount of activity appeared in the A hemoglobin fraction following incubation. The small amount of globin activity which did appear is commensurate with the slight chromatographic overlapping and incomplete separation of the F and A hemoglobins. That the exchange phenomenon involves the entire heme group, and not the iron alone, is shown from an experiment in which both the heme and the globin of ferrihemoglobin F were labeled with 2-C^{14}-glycine. Considerable exchange of heme label occurred during the incubation, but again very little activity was recovered in the globin fraction of A hemoglobin.

Table 2 shows the rate of heme exchange between corresponding ligands of hemoglobins F and A. After 5 hr incubation, only the ferrihemoglobin showed significant heme exchange, there being little or no transfer of radioactivity with the oxyhemoglobin, deoxyhemoglobin, ferrihemoglobin-cyanide, or carboxyhemoglobin forms. It is uncertain whether the small amount of "exchange" noted with carboxyhemoglobin and cyanmethemoglobin represents actual transfer of heme groups or a small amount of contamination due to imperfect separation of F and A hemoglobins; the somewhat greater exchange with oxyhemoglobin may have resulted in part from a small amount of autoxidation during the incubation. When ferrihemoglobin was bound to human serum haptoglobin, the transfer of labeled hemes was completely prevented. Thus, the phenomenon of heme exchange appears to depend upon the presence of free ferrihemoglobin.

It was necessary to show that heme exchange was not peculiar to ferrihemoglobin engendered by ferricyanide, although that agent has been shown to have a highly

TABLE 1

TRANSFER OF RADIOACTIVITY FROM LABELED FERRIHEMOGLOBIN F TO FERRIHEMOGLOBIN A

Radioactive label	Spec. Act.* after 16 Hr Incubation (cpm/mg hemoglobin) F	A
Fe^{59}	77 ± 4	69 ± 4
C^{14}-leucine	540 ± 54	26 ± 11
2-C^{14}-glycine		
Heme	509 ± 27	367 ± 23
Globin	842 ± 87	77 ± 8

* Mean ±SD of neighboring fractions containing more than 0.5 mg hemoglobin/ml.

TABLE 2

EFFECT OF VARIOUS LIGANDS ON THE TRANSFER OF RADIOACTIVITY FROM Fe^{59}-LABELED HEMOGLOBIN F TO HEMOGLOBIN A

Form of hemoglobins during incubation	Heme exchange* (%)
Oxyhemoglobin†	7.5
Deoxyhemoglobin	5.5
Ferrihemoglobin	75.5
Ferrihemoglobin cyanide	3.1
Carboxyhemoglobin	3.3

* After incubation at 37°C, pH 7.18, for 5 hr.
† A small amount of autoxidation occurred during incubation. The ferrihemoglobin concentration at the end of incubation was 7%.

specific action on heme under the conditions employed.[5, 6] A hemolysate of red cells from a patient with congenital methemoglobinemia, due to a deficiency of the NADH-linked methemoglobin reductase, contained 15 per cent methemoglobin. The rate of heme exchange from this "spontaneous" ferrihemoglobin was found to be quite similar to that of a comparable ferrihemoglobin:oxyhemoglobin mixture prepared with ferricyanide.

Whereas in mixtures of oxyhemoglobin the hemes exchanged little, if at all, considerable exchange occurred with mixtures of ferrihemoglobin and oxyhemoglobin. For example, equal amounts of labeled ferrihemoglobin F and unlabeled oxyhemoglobin A showed 22 per cent exchange after a standard 100-min incubation, compared to a mixture of the completely oxidized hemoglobins, which exchanged 51 per cent of its hemes. Similarly, a mixture of labeled oxyhemoglobin F and unlabeled ferrihemoglobin A exchanged 35 per cent. Relatively little spontaneous autoxidation occurred during these incubations, but there was an exchange of electrons between the unlike hemoglobins of a magnitude similar to, or greater than, the magnitude of heme exchange.

The rate at which the hemes of ferrihemoglobins A and F exchanged was quite consistent. In nine 100-min incubations at pH 7.18, ionic strength 0.09, 37°C, done on separate days, the mean per cent exchange was 46.2, $\pm$ 1 standard deviation of 4.1 per cent. This rate was the same, regardless of which of the two hemoglobins was initially labeled. Furthermore, the rate was independent of hemoglobin concentration over a ninefold range. The rate of exchange between ferrihemoglobins F and A appeared to follow first-order kinetics. The exchange rate of hemoglobin labeled only in the alpha chain was considerably less than that of the uniformly labeled compound. Heme exchange between β chains of A and γ chains of F was about four times as rapid as between α chains of A and α chains of F. Detailed kinetic analyses will appear in a subsequent report and will include estimations of the heme:globin dissociation constants for alpha and non-alpha chains.

Heme exchange increased with temperature over the range studied of 4–40°C; the heat of activation was calculated as 20 cal/mole. When the pH of the incubation buffer was increased from 6.4 to 7.7, the rate of heme exchange was enhanced 52 per cent. On the other hand, heme exchange was affected little by varying the ionic strength of the media from 0.008 to 0.320.

Discussion.—Our results indicate that the reversible oxidation of human hemoglobin to ferrihemoglobin permits free heme exchange among intact molecules. Heme exchange is almost as rapid in mixtures of methemoglobin and oxyhemoglobin, possibly involving an exchange of electrons. Since free ferriheme associates

very rapidly with free globin,[7] exchange among intact molecules presumably reflects the extent of dissociation. This lability is probably related to the ionic nature of the bond between globin and ferriheme. Ligands appear to stabilize methemoglobin by transforming the heme:globin linkage into a more covalent bond, as indicated by a reduction of magnetic moment. Although deoxyhemoglobin is similar in this respect, it differs from ferrihemoglobin in tertiary structure and in a number of physical and chemical properties,[8] including its increased resistance to salt denaturation and to conversion to "parahematin."[9] In the intact red cell, where methemoglobin is constantly being formed and then actively reduced, an exchange of hemes among molecules must proceed continuously. In disorders involving methemoglobinemia, heme exchange among molecules must be extensive. Hemoglobin released from red cells into plasma or tissues undergoes autoxidation to methemoglobin, resulting in heme exchange and, in plasma, heme transfer to albumin, which is present in considerable excess. The very condition which permits heme exchange among hemoglobin molecules, i.e., the presence of free ferrihemoglobin, also allows the transfer of heme to albumin, resulting in the formation of methemalbumin.[10] In studies presented in preliminary form elsewhere,[10] we have found that ferrihemes, bound to human albumin, exchange reversibly with those of methemoglobin, the affinity of albumin being about one ninth that of globin. By blocking the dissociation of heme from methemoglobin, haptoglobin may help prevent the indiscriminate loss of heme, and thereby, of its iron.

The possibility of exchange of hemes among apoproteins was raised by Yčas and Drabkin[11] on the basis of studies of C^{14}-labeled cytochromes in yeast. Rossi-Fanelli and Antonini[12] observed that both horse apomyoglobin and apohemoglobin were able to remove hemes from *Aplysia* (sea hare) myoglobin. They, and Banerjee,[13] found that horse apomyoglobin would accept hemes from horse methemoglobin in equimolar concentration under physiological conditions. Apparently, horse apomyoglobin has a stronger heme:globin linkage than does horse globin. We have found that hemes from human metmyoglobin do not transfer to albumin nearly as readily as do those from methemoglobin. Teale[14] explored the possibility of heme transfer from hemoglobin to free globin by use of a fluorescent label, dimethyl aminonaphthalene sulfonamide, which is quenched in the presence of free heme groups; heme:globin dissociation was inferred by an increase in fluorescence on the addition of free globin. The equilibrium studies cited above[12-14] all require the presence of an artificially prepared apoprotein which acts as a heme receptor. Our studies describe the exchange of hemes between intact hemoglobin molecules under physiological conditions.

Controlled and reversible heme dissociation and exchange may be a feature of the interactions of various heme-containing enzymes that are involved in oxidation reduction and may be critical to the feedback mechanisms controlling the rate of heme synthesis. Certainly some previous studies depending upon the use of heme labels may require reinterpretation. The experimental approach described may be utilized in the preparation of a wide range of labeled hybrids, and may provide further information about the nature of the heme:globin bond.

The technical assistance of Miss V. Field and the editorial assistance of Miss W. M. Sheldon are greatly appreciated. The authors also wish to express their gratitude to the staff of the De-

partment of Gynecology and Obstetrics, Boston City Hospital, for their cooperation in procuring the specimens of umbilical cord blood.

* Supported in part by USPHS grants HE-07652 and T1-AM-5391.

† NIH Research Career Development Award K3-HE-3943 from the National Heart Institute.

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