EFFECT OF LIGANDS ON ALKALINE DENATURATION OF FETAL HEMOGLOBIN AND ISOLATED GAMMA SUBUNITS

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SUMMARY

The rates of alkaline denaturation of hemoglobin A, hemoglobin F, and isolated alpha, beta, and gamma subunits have been measured as a function of the ligand state of the heme iron. Under all conditions hemoglobin A and its isolated subunits were completely denatured before the initial measurement was made. A similar result was obtained with the high spin ferric (met) forms of hemoglobin F and isolated gamma subunits. However, in the low spin ferric (cyanmet) and liganded ferrous (oxy, carbonmonoxy) forms, hemoglobin F was partially resistant to alkaline denaturation and this resistance could be attributed to the presence of gamma subunits. In addition, the alpha subunits of hemoglobin F are more stable than those of hemoglobin A due to their association with gamma subunits. These results suggest that the above liganded forms of hemoglobin F have similar structures and this structure is different from that of the same derivatives of hemoglobin A.

A major difference between hemoglobin A and hemoglobin F is found in their respective rates of alkaline denaturation with hemoglobin F being much more stable to alkaline conditions than hemoglobin A (1,2). This difference has been utilized in estimating the amount of hemoglobin F present in human red cell extracts, and the relative resistance of hemoglobin F to alkaline conditions has been attributed to the presence of gamma subunits (2,3). Recently it has been shown that isolated gamma subunits in the cyanmet form are substantially more stable than hemoglobin A, though less stable than hemoglobin F (4). In the present study the alkaline denaturation characteristics of hemoglobin A, hemoglobin F, and isolated alpha, beta, and gamma subunits have been examined in the presence of different ligands in an effort to determine the basis of the difference in alkaline stability observed between hemoglobin F and hemoglobin A.

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MATERIALS AND METHODS

Hemoglobin A was isolated from red cells by the procedure of Drabkin (5). Cord blood was kindly provided by the Parkland Hospital Blood Bank and the hemoglobin isolated in the same manner as above. Hemoglobin F was separated from hemoglobin A by the chromatographic method of Huisman and Meyerling (6). Purity of the hemoglobin F was established by disc gel electrophoresis carried out as described by Ornstein and Davis (7,8).

Subunits of hemoglobin A were isolated by ion exchange chromatography following treatment with p-mercuribenzoate (PMB) (9), while gamma subunits were isolated from hemoglobin F by the PMB method of Noble (10). The PMB was removed from all subunits by emulsification with 1-dodecanethiol (11). The number of free sulfhydryl groups on each isolated subunit after dodecanethiol extraction was determined by the PMB titration method of Boyer (12).

The rate of alkaline denaturation was measured by a modification of the method of Huehns, et al. (2). Hemoglobin samples were diluted with water to obtain absorbancies of about 0.9 at the wavelengths given below. After placing 0.12 ml of 2N NaOH in a spectrophotometer cuvette, 3.0 ml of the sample to be studied was added. Absorption readings were taken at various times after sample addition. The initial absorbance was measured on a control sample diluted with 0.12 ml of water in place of the NaOH. The final absorbance, due to the completely denatured hemoglobin derivative, was measured on a sample containing 0.12 ml of NaOH which had been incubated at 37°C. for 15 minutes. Denaturation of oxy derivatives was followed at 415 nm, cyanmet and carbonmonoxy derivatives at 420 nm, met gamma subunits at 410 nm, methemoglobin F at 407 nm, and methemoglobin A at 404 nm. These wavelengths represent the maximum absorbance difference between native and completely denatured samples. Spectrophotometric measurements were carried out with a Coleman 124 Perkin-Elmer double beam spectrophotometer at room temperature.

RESULTS AND DISCUSSION

The results of the alkaline denaturation studies are shown in Fig. 1. In

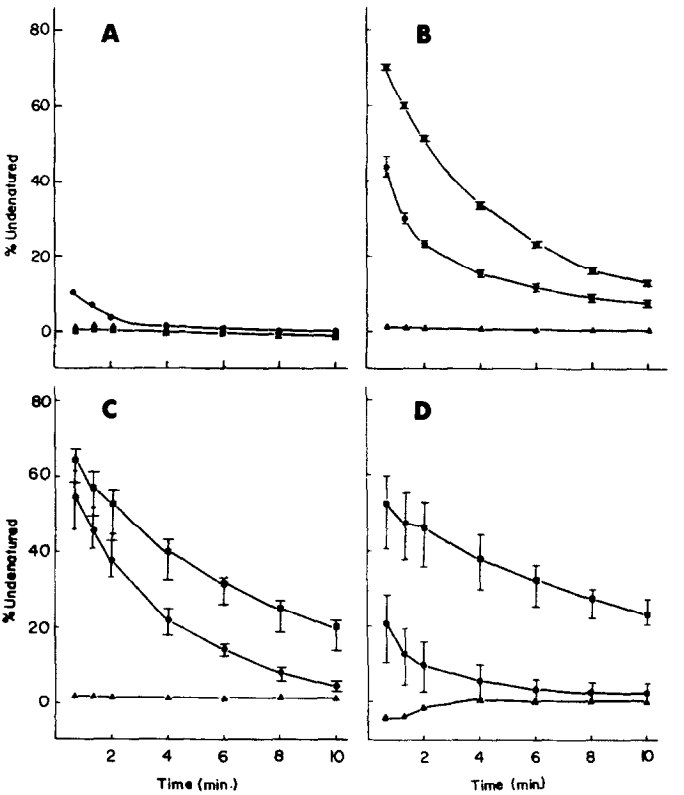

Figure 1: Rates of alkaline denaturation of hemoglobin A, hemoglobin F, and isolated gamma subunits. The percent of denatured hemoglobin present (determined as described in the text) has been plotted against time. Hemoglobin A is represented by the solid triangles (▲), hemoglobin F by the solid squares (■), and isolated gamma subunits by the solid circles (●). A) alkaline denaturation rates of met (ferric) forms, B) alkaline denaturation rates of the cyanmet forms, C) alkaline denaturation rates of the carbonmonoxy forms, and D) alkaline denaturation rates of the oxy forms. Each point is the average of at least five separate measurements and the error bars show the range obtained at each time interval. All measurements were made at room temperature.

Fig. 1-A, the rate of alkaline denaturation of the ferric (met) forms of hemoglobin A and F and isolated gamma subunits are seen. Under the conditions employed, no appreciable alkaline stability is observed with any sample. In Fig. 1-B, the effect of cyanide as the heme ligand on the alkaline denaturation rate of the above ferric forms is seen. In the case of both hemoglobin F and isolated gamma subunits, the presence of cyanide substantially retards the denaturation process while having no measurable effect on that of hemoglobin A. In Fig. 1-C, it is seen that reduction of the heme iron in the presence of carbon monoxide affords no greater resistance to the alkaline denaturation of

hemoglobin F and isolated gamma subunits than does cyanide. In Fig. 1-D, the effect of oxygen as a ligand in place of carbon monoxide is seen. The rates of denaturation are faster in this case, especially that of isolated gamma subunits. In all forms, hemoglobin A was completely denatured before the first reading was taken (20 seconds), as were all forms of isolated alpha and beta subunits. Stopped-flow measurements showed the alkaline denaturation rate of methemoglobin F to be slower than that of methemoglobin A, but both reactions had half-times of less than one second.

The rate of loss of absorbance under alkaline conditions is a function of the ligand state of the hemoglobin iron atoms. In the ferric state, water is at the sixth coordination position of the heme iron and all samples are very unstable to alkaline conditions. When water is replaced by cyanide, a significant decrease in the rates of denaturation of both hemoglobin F and isolated gamma subunits is observed. However, the replacement of water as a ligand is not sufficient to induce alkaline stability. Fluoride had no effect on the alkaline denaturation rate of methemoglobin F. Fourier difference synthesis of x-ray data from metmyoglobin and its cyanide derivative show that changes in tertiary structure take place upon ligand binding (13,14). The alkaline stability demonstrated by the cyanmet form of isolated gamma subunits may also reflect a change in tertiary structure upon ligand binding. Such a change in structure would be the result of a ligand induced transition of the heme iron from the high spin ferric form to the low spin ferric form. Fluoride maintains the high spin ferric form and therefore does not change the alkaline denaturation rate. If similar changes in structure take place in the isolated alpha and beta subunits of hemoglobin A upon ligand binding, they do not result in a measurable increase in alkaline stability. The fact that hemoglobin F is even more stable than isolated gamma subunits suggests that similar ligand-induced conformational changes may take place within the tetramer and that they are better maintained within the tetramer, leading to greater stability.

The oxy and carbonmonoxy forms of isolated gamma subunits and hemoglobin

F are seen to have similar stabilities to those of the cyanmet forms, although the oxy forms are somewhat less stable. The decreased stability of the oxy form may be due to facilitated autoxidation of the ferrous heme iron under alkaline conditions. However, the presence of cyanide during denaturation of oxyhemoglobin F did not increase the alkaline stability. In the case of hemoglobin A, the oxy, carbonmonoxy, and all ferric forms are thought to have similar or identical three-dimensional structures (15). It would appear from the alkaline denaturation data in Fig. 1 that the liganded ferrous (oxy and carbonmonoxy) and the low spin ferric (cyanmet) forms of hemoglobin F also have a similar structure in solution. This structure, however, is different from that of the same derivatives of hemoglobin A. In addition, the high spin ferric form of hemoglobin F, having either water or fluoride as the sixth ligand, is structurally different as measured by alkaline stability.

There are thirty-nine amino acid sequence differences between beta and gamma subunits (16). While many of these substitutions are of the conservative type, they must be sufficient to cause isolated gamma subunits in the liganded ferrous and low spin ferric forms to assume a conformation which is more stable to alkaline conditions than either isolated alpha or beta subunits. The fact that alkaline stability is increased in the hemoglobin F tetramer suggests that the denaturation of the alpha subunits is retarded when they are combined with gamma subunits. Of the eighteen beta chain residues involved in the hemoglobin A $\alpha_1 \beta_1$ contact point, five of these residues are replaced in the gamma subunit. It is possible that the increased stability of alpha subunits in the hemoglobin F tetramer as compared to the hemoglobin A tetramer is the result of interactions which take place between subunits at this contact point.

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