Hemoglobin Richmond

SUBUNIT DISSOCIATION AND OXYGEN EQUILIBRIUM PROPERTIES*

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In hemoglobin Richmond (β102 → Lys), amino acid substitution has occurred at the same site as the mutation in hemoglobin Kansas (β102 Asn → Thr), a variant with very low oxygen affinity. Although hemoglobin Richmond has been shown to have increased tetramer-dimer dissociation, its oxygen affinity has been inferred to be normal from studies on hemolysates of carriers.

We have isolated hemoglobin Richmond and have further studied its properties. We confirm that the oxygen affinity of pure hemoglobin Richmond under conditions similar to those found in vivo is normal. However, the Bohr effect of the variant hemoglobin is markedly abnormal. Its oxygen affinity is low at high pH and high at low pH, relative to hemoglobin A. The tetramer-dimer equilibrium displays a strong pH dependence such that protons promote dissociation.

A model is presented in which the structural change in hemoglobin Richmond results in low oxygen affinity, like hemoglobin Kansas. However, the close linkage between tetramer-dimer dissociation and proton concentration seen with hemoglobin Richmond results in normal oxygen affinity at intracellular pH and hemoglobin concentration, and carriers display no hematological abnormalities.

Of all of the human hemoglobin mutants known to occur at the α2β2 interface (1-13), hemoglobin Richmond (β102 Asn → Lys) is the only one whose oxygen affinity has been considered to be normal (1). It has a dissociation constant for the tetramer-dimer equilibrium which is approximately 5 times that of hemoglobin A at neutral pH, and asymmetrical hybrids (αβββRichmond) can be detected in hemolysates. The oxygen equilibrium of purified hemoglobin Richmond has not actually been measured, because formation of hybrids leads to difficulty in isolation. Rather, stripped, concentrated hemolysates in phosphate buffer were studied by Efremov et al., and oxygen equilibrium, cooperativity, and Bohr effect of hemoglobin Richmond were inferred to be normal (1).

Hemoglobin Kansas (β102 Asn → Thr) is produced by amino acid substitution at the same site as that in hemoglobin Richmond. Both abnormalities involve Asn G48, which normally forms one of the hydrogen bonds that stabilize the oxy conformation of the molecule (14). The crystal structures of deoxy-Kansas and Richmond have been studied at relatively low resolution; both are abnormal, but the changes in the former are much more marked, affecting the heme contacts as well as the α2β2 interface (15). Although understanding of structure-function relationships in hemoglobin is incomplete, it seemed anomalous that the functional properties of hemoglobin Richmond were normal.

We have recently encountered a second American Negro kindred in which several family members are carriers of hemoglobin Richmond. We have been unable to detect clinical abnormalities in these individuals and have confirmed the earlier report of normal oxygen affinity of whole blood. We have been able to purify the abnormal component, and have shown that its oxygen affinity, cooperativity, and subunit dissociation are, in fact, abnormal.

METHODS

Whole Blood Oxygen Equilibria—Blood was collected in heparin, immediately cooled on ice, and oxygen equilibria were measured within 4 hours. Three milliliters of blood were placed in each of several kugeltonometers (Eschweiler & Co., Kiel) and equilibrated at 37° for 20 min with analyzed mixtures of oxygen (2.18 to 6.13%) and nitrogen of varied proportions, each of which contained 5.6% CO2 (Air Products, Emmaus, Pa.). Preliminary experiments indicated that equilibration was achieved after this time.

The pH of each sample was measured using a microelectrode (Radiometer, Copenhagen), and the oxygen saturation was determined with an IL Co-Oximeter (Instrumentation Laboratories, Lexington, Mass.) which had been slightly modified to permit direct aspiration of blood samples from the tonometers without the use of syringes. The ρO2 values were corrected to pH 7.4 using an assumed Bohr factor

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The abbreviations used are: DPG, 2,3-diphosphoglyceric acid; bis-Tris, bis(2-hydroxyethyl)iminotri(hydroxymethyl)methane; ρO2, oxygen tension; ρO2* the oxygen tension at which half-saturation of
Isolation of Hemoglobin Richmond—Red cells collected in sodium citrate or heparin were washed three times in 0.15 M NaCl and lysed with an equal volume of distilled water. After sedimentation of the cellular debris by centrifugation, the hemoglobin solutions were diluted with an equal volume of 0.02 M phosphate buffer, pH 7.2, saturated with CO. Columns of Bio-Rex 70 (Bio-Rad, 2.5 × 40 cm) were equilibrated with the CO-saturated buffer for a least 4 days at room temperature prior to use, and then about 1 g of carboxyhemoglobin was applied. Hemoglobin Richmond remained bound to the top of the column, and after recovery of hemoglobin A, it was eluted as a concentrated band with distilled water. After further concentration to 10 g/ml by ultrafiltration using dialysis tubing at 4°C, less than 5% methemoglobin could be detected, and the solution was stored as carboxyhemoglobin at 4°C for up to 3 weeks until use.

Identification of Hemoglobin Richmond—Electrophoresis of fresh hemolysates was carried out using starch gel (18), citrate agar (19), and Mylar-backed sheets of cellulose acetate (Helena Laboratories, Beaumont, Tex.). Globin was prepared from purified hemoglobin Richmond according to the method of Rossi-Fanelli et al. (20) and component polypeptide chains were isolated by the method of Ciec et al. (21). The resultant globin peaks were aminoethylated, exchanged through Sephadex G-25 (Pharmacia, Piscataway, N. J.) into formic acid (0.1 mg/ml), and lyophilized. They were then digested with trypsin, fingerprinted, and the tryptic peptides were isolated and their amino acid compositions were determined according to methods previously described (6).

Hemoglobin Oxygen Equilibria—Carboxyhemoglobins were "stripped" of organic phosphates by passage through a column of Sephadex G-25 equilibrated with 0.05 M bis-Tris/0.1 M NaCl, pH 7.2, at 4°C, and saturated with CO. The CO was subsequently removed by a modification of the photolysis method described by Kilmartin (22). The bis-Tris NaCl buffer was then added to adjust pH with only a minor change in ionic strength or buffer composition.

Oxygen equilibrium was measured at 10°C using tonometers similar to those described by Rossi-Fanelli (23). For dilute solutions (0.01%) 420 nm was found to be isobestic for oxy- and deoxyhemoglobins, and the ratio A420/A578 was taken as a measure of saturation. For concentrated solutions (1 g/100 ml), the ratio A412/A578 was used, and for intermediate concentrations (0.1 g/100 ml), A410/A570, and A417/A572 were used as described earlier (6). For experiments in which DPG was added to "stripped" hemolysates, the pentacyclohexylammonium salt was converted to the free acid with Dowex 50 as described by Benesch et al. (24). DPG concentrations were measured according to Nygaard and Rorth (25), and not more than 1/5 volume was added to each sample. Data was corrected to pH 7.2 (26), and the best straight line was drawn through the points by visual inspection. Since a line so derived departs from linearity at the extremes of saturation (27), added weight was given to the points nearest 50% saturation. Hill's coefficient, n, and pKo were obtained in this graphic manner.

Tetramer-Dimer Equilibrium of Hemoglobin Richmond—Estimates of the relative association constants of liganded hemoglobins A and Richmond were obtained by a modification of the difference gel filtration procedure of Gilbert (28). Sephadex G-100 was equilibrated with 0.05 M Tris-C/0.1 M NaCl, pH 7.2, in a jacked column (95 × 1.6 cm, Pharmacia) and was operated at 4°C with a flow rate of 6.9 ml/hour. The buffer was flushed daily with CO, a flow adaptor (A16, Pharmacia) was used for sample application, and the effluent was monitored with a Gilford spectrophotometer (Gilford, Oberlin, Ohio).

A single column was operated continuously and was used to obtain all of the data reported here. When the pH of the buffer was changed, 4 days were allowed for equilibration with the new buffer. The column was calibrated under each set of conditions with blue dextran (Phar-macia), sperm whale myoglobin, and bovine serum albumin (Worthington). This was achieved by loading sufficient sample so that a plateau in the effluent tracing resulted, and the elution volume was determined by analysis of the leading boundary between buffer and protein.

Concentrated hemoglobin solutions were exchanged into appropriate CO-saturated buffers using Sephadex G-25 (coarse) columns, then diluted to the desired concentration in the same buffer. Enough of the sample was loaded onto the G-100 column so that a plateau in the absorbance of the effluent was achieved. The inflow was then abruptly switched to a second sample with the same hemoglobin concentration as the first, and an equal amount was loaded in tandem.

The total amount of hemoglobin loaded was calculated from its absorbance and the flow rate of the column. The area of deviation from the plateau at the junction of the two tracings was determined graphically as a fraction of the total load, and the elution volume difference was obtained using the protein concentration. The value, ΔV, was then related to the degree of dissociation of hemoglobin A (28-31). The association constant for hemoglobin A, 250 g/dl, was taken from Chiancone et al. (21), and it was assumed to be constant over the pH interval studied (90, 32).

RESULTS AND DISCUSSION

Identification of Hemoglobin Richmond—The electrophoretic properties of hemolysates were identical to those reported by Efremov et al. (1). The presence of an abnormal hemoglobin component could be clearly demonstrated by electrophoresis at pH 6.0 in citrate agar. After isolation of this component, tryptic peptides corresponding to residues 1896 to 102, 1013 to 104, and 896 to 104 were analyzed and demonstrated the substitution of lysine for asparagine 1012 (G4) of hemoglobin Richmond.

The oxygen equilibrium of whole blood from a carrier was normal (P50 = 26.5 mm Hg) after correction to a plasma pH of 7.4. The Bohr effect was assumed to be normal in making this correction; the consequent error was very small, since the pH range for the various samples was 7.39 to 7.41.

Oxygen Equilibrium of Purified Hemoglobin Richmond—The curves relating log P50 and pH for stripped hemoglobins A and Richmond intersect at pH 7.4 (Fig. 1). At pH 7.2 (approximately intracellular pH), in the absence of DPG, hemoglobin Richmond had a slightly higher affinity for oxygen than did hemoglobin A, while in the presence of DPG (DPG/hemoglobin = 2), the equilibria were indistinguishable. At pH above 7, DPG had a greater, and below 7, a smaller, effect on hemoglobin Richmond than on hemoglobin A. Like hemoglobin A (33), the Bohr effect of hemoglobin Richmond is enhanced by DPG. Hill's coefficient, n, was decreased below pH 7.2 (Fig. 2) in experiments with hemoglobin Richmond.

Tetramer-Dimer Equilibrium—Hemoglobin Richmond appeared to be less associated than hemoglobin A at all pH values studied, and the association constant (K) appeared to vary directly with pH: K = 20 dl/g at pH 6.7, 80 at pH 7.2, and 150 at pH 7.7. These data suggest that at pH 7.2 under the conditions shown in the experiment in Fig. 1, hemoglobin Richmond is about 3 times more dissociated than hemoglobin A. This is in good agreement with the results of Efremov et al. (1).

Dilution (Table I) appeared to have a greater effect on oxygen affinity, and perhaps cooperativity, of hemoglobin Richmond than of hemoglobin A. Moreover, the magnitude of the effect appeared to be pH-dependent, suggesting a relation between tetramer-dimer dissociation and proton binding (34).

Model for Function of Hemoglobin Richmond—The peculiar change in oxygen affinity of hemoglobin Richmond with pH, relative to that of hemoglobin A (Fig. 1), may be produced by two opposing effects of the amino acid substitution. The relativity low affinity seen at high pH may reflect inherently low oxygen affinity of the R structure of hemoglobin Richmond, similar to the low affinity of the R structure of hemoglobin Kansas (35). The high affinity seen at low pH may be produced by an allosteric mechanism, since an increased H+ concentration favors tetramer-dimer dissociation which, in turn, shifts the R-T equilibrium toward R (36-40).

*J. V. Kilmartin, personal communication.
FIG. 1. Oxygen equilibria of hemoglobin A (O) and Richmond (A) as a function of pH. Each point represents a single experiment. Purified hemoglobins were "stripped" of organic phosphates, and studied before (O, A) or after (O, A) the addition of DPG ($3 \times 10^{-4}$ M). Hemoglobin concentration was $1.5 \times 10^{-4}$ M (tetramer).

The model can explain the normal oxygen affinity of whole blood and the lack of clinical consequences in carriers. That this physiological property is normal because of a complex interaction of opposing molecular derangements demonstrates the critical nature of the $\alpha \beta_5$ interface.

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**REFERENCES**


In analyzing the dissociation of hemoglobins at low pH, Wyman (34) suggested a model for linkage between dissociation and proton binding such that the dissociation products (dimers) together bind more protons than tetramers. The increased proton-linked dissociation which we see in oxyhemoglobin Richmond may be an augmentation of such an effect. Since the pK of the new lysine at position G4$\beta$ is too high to be responsible for increased proton binding, interaction with some neighboring group must be invoked. Model building does not provide a simple answer as to what this group(s) might be.2
Hemoglobin Richmond. Subunit dissociation and oxygen equilibrium properties.
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