Isolation and Functional Characterization of Hemoglobin Casper: β106(G8)Leu→Pro†

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ABSTRACT: Hemoglobin Casper (β106Leu→Pro) can be separated from hemoglobin (Hb) A by isoelectric focusing on polyacrylamide gel. This abnormal hemoglobin was estimated to be 30% of the total by both isoelectric focusing and heat lability kinetics. Its oxygen equilibrium curves indicate a high oxygen affinity, low degree of subunit interaction, and a decreased Bohr effect. Mixtures of Hb Casper and Hb A appear to bind oxygen as if no hybrid molecules exist.

Hemoglobin Casper or Southampton has been recognized in five unrelated individuals, all of whom represent sporadic cases presumably due to new mutations (Jones et al., 1972; Hyde et al., 1972; Idel'son et al., 1974; T. Bradley, personal communication). The structural abnormality of this hemoglobin is the substitution of leucyl residue 106 (G8) of the β chain by a prolyl residue (Jones et al., 1972; Koler et al., 1973; Hyde et al., 1972). Similar to some other unstable hemoglobin (Hb)[1] variants with neutral substitutions, Hb Casper cannot be separated from Hb A by starch gel electrophoresis or by column chromatography on Amberlite IRC 50 (Labie et al., 1972) or DEAE-Sephadex (Wajcman et al., 1973). For structural characterization, the abnormal hemoglobin was isolated from hemoglobin A by heat precipitation or by treatment with *p*-mercuribenzoate (Jones et al., 1972). Until the present study it had not been possible to obtain the pure abnormal hemoglobin in an undenatured form. Its general functional properties were deduced from measurements done on intact erythrocytes or whole hemolysates.

In this paper we describe the isolation of pure, native hemoglobin Casper by isoelectric focusing on polyacrylamide gels. Using this method on a preparative scale sufficient amounts of the abnormal hemoglobin were obtained to perform functional studies which are also described.

Experimental Procedures

Hemolysates were prepared by routine procedures (Drabkin, 1946).

The kinetics of heat lability at 65°C were studied according to the methods of Rieder (1970) with slight modifications (Wajcman et al., 1973).

Electrofocusing was performed on 4% polyacrylamide gels containing 2% Ampholine (LKD 6–8) as described by Drysdale et al. (1971). The analytical separations were made in 5 × 130-mm tubes using a commercial apparatus from Hoefer Scientific Instruments. In order to obtain larger quantities of abnormal hemoglobin, the electrofocusing was modified by utilizing tubes of i.d. 15 × 140 mm onto which 30 mg of hemoglobin in 5% saccharose was applied. The separations were obtained at 8°C using 400 V for 6 hr. After the electrofocusing was obtained, the gels were sliced and the bands eluted directly in a Visking tube by electrophoresis in Tris–EDTA–borate buffer at pH 8.8 (Suzuki et al., 1973). The scanning of the analytical gels was performed at 540 nm using a Gilford spectrophotometer. No staining was necessary because the color of the hemoglobin was sufficient for detection and quantitation.

Oxygen equilibria were studied by the spectrophotometric technique of Benesch et al. (1965) as modified by Bellingham and Huehns (1968). Measurements using intact red blood cells were made in isotonic phosphate buffer at pH 7.1 and 37°C. The hemolysates were freed of organic phosphates by gel chromatography on Sephadex G-25 as described by May and Huehns (1972). The oxygen affinities of the hemolysate and pure component were studied in 0.05 *M* bis-tris buffer at 25°C. The effect of pH on the oxygen equilibria was studied at 25°C in 0.05 *M* bis-tris–0.01 *M* NaCl buffer below pH 7.5 and in 0.05 *M* Tris-HCl–0.1 *M* NaCl above this pH. The action of organic phosphates on hemolysates and pure components was studied by adding known amounts of neutralized 2,3-DPG or IHP into the tonometer.

Intra-erythrocytic 2,3-DPG was assayed by Beutler's modification of Krimsky's method (Beutler et al., 1969). The regulatory action of organic phosphates was studied in intact cells by measuring the oxygen affinity at different intracellular concentrations of 2,3-DPG. Its concentration was increased by the method of Deuticke et al. (1971) as follows: the cells were incubated under nitrogen at pH 7.35 in an isotonic solution containing inosine (10 m*M*), pyruvate (10 m*M*), and inorganic phosphates (75 m*M*).

Spectrophotometric studies were performed in 0.15 *M* phosphate buffer (pH 6.5) with a Beckman Acta III spectrophotometer.

Results

The percent of unstable hemoglobin present in the whole hemolysate was estimated by heat stability kinetics to be

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[1] Abbreviations used are: IHP, inositol hexaphosphate; 2,3-DPG, 2,3-diphosphoglycerate; Hb, hemoglobin; HbO_2, oxyhemoglobin; bis-tris, 2,2-bis(hydroxymethyl)-2,2′,3″-nitriloethanol; P_{50}, oxygen pressure at which hemoglobin is 50% saturated with oxygen.

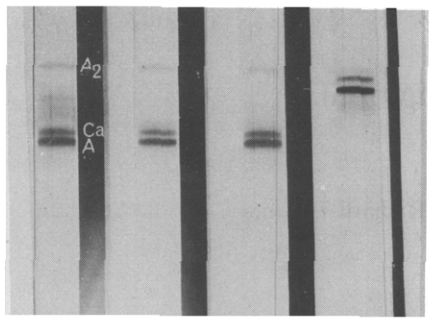

FIGURE 1: Isoelectric focusing of hemolysate containing Hb Casper on polyacrylamide gel. (a) Total lysate containing hemoglobin Casper and three minor bands due to partial oxidation of hemoglobins. (b) Testing for the presence of deheminized hemoglobin in lysate by addition of potassium cyanide and cyanhemin which convert globin and methemoglobin to cyanmethemoglobins which electrofocus with oxyhemoglobins. (c) Transforming ferrihemoglobin to cyanmethemoglobin by addition of potassium cyanide to lysate. When compared to gel b indicates absence of deheminized hemoglobin. (d) Fully oxidized lysate by addition of a slight excess of potassium ferricyanide.

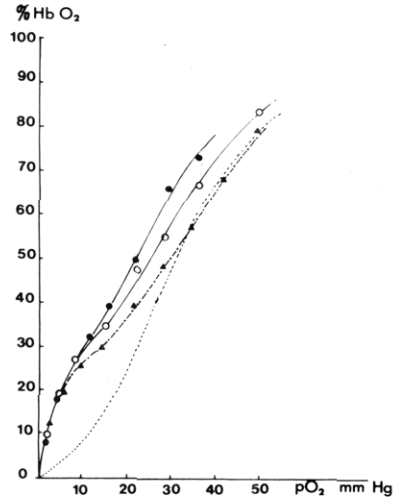

FIGURE 2: Oxygen equilibrium curves of red blood cells suspended in isotonic phosphate buffer (pH 7.1) at 37°C. The intra-erythrocytic 2,3-DPG concentration was 20 μmol/g of Hb in the starting sample of red cells containing Hb Casper (●—●). After incubation in an inosine, pyruvate, and phosphate medium it was raised to 26 μmol/g of Hb (○—○) and to 32 μmol/g of Hb (▲- -▲). Normal red cell control with a 2,3-DPG content of 15 μmol/g of Hb (- - -).

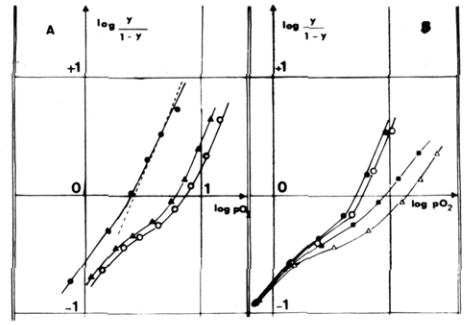

FIGURE 3: Oxygen equilibrium curves of lysates. Hill plots of oxygen equilibrium measurements where Y represents the fraction of oxyhemoglobin. In all cases the hemoglobin concentration was 2×10^{-5} M. (A) Measurements in bis-tris 0.05 M pH 7.15 buffer at 25°C: (●—●) stripped lysate containing 30% hemoglobin Casper; (▲—▲) same sample after addition of 2,3-DPG to a final concentration of 26 μM; (○—○) same sample with final 2,3-DPG concentration of 52 μM; (- - -) normal stripped hemoglobin A lysate. (B) Measurements in 0.05 M bis-tris–0.1 M NaCl (pH 7.15) buffer at 25°C: (●—●) stripped lysate containing 30% hemoglobin Casper; (○—○) same sample after addition of 2,3-DPG to a final concentration of 43 μM; (■—■) same sample after addition of IHP to a concentration of 16 μM; (△—△) same sample after addition of IHP to a concentration of 30 μM.

approximately 30%. The electrofocusing on polyacrylamide gel demonstrated the presence of an abnormal hemoglobin band which focused at a pH_i 0.1 unit above the band of hemoglobin A as seen in Figure 1. Loss of heme from the abnormal hemoglobin appeared to be negligible although there was some partial oxidation as demonstrated by comparing gels of samples with and without added potassium cyanide and cyanhemin. By scanning the gel the percent of the abnormal hemoglobin was also estimated to be 30%.

Intact Red Blood Cells. Hemoglobin Casper was obtained from samples of whole blood preserved in Alsever's solution and kept in ice for the 4 days required for shipment between Portland and Paris. When received, the 2,3-DPG concentration was still high at 20 μmol/g of Hb compared to 14.7 ± 1.0 μmol/g of Hb for normal fresh cells. It was less than a fresh sample measured earlier at 33.0 μmol/g of Hb (Jones et al., 1972). The oxygen equilibrium curves of red blood cell suspensions are shown in Figure 2. The P_{50} of the red blood cells suspended in isotonic phosphate buffer at pH 7.1 was 22 mm compared to 32 mm for normal cells containing an identical amount of 2,3-DPG. The oxygen equilibrium curve of the abnormal cells was biphasic with a greatly increased oxygen affinity at the low P_{O_2} values.

After incubation of the abnormal cells in an inosine, pyruvate, and phosphate medium, the intra-erythrocytic content of 2,3-DPG was increased but only a slight shift of the oxygen affinity curve was observed. The curves were clearly modified above but not below 25% oxyhemoglobin.

Whole Lysate. The oxygen equilibrium curve of the lysate containing hemoglobin Casper which had been stripped of organic phosphates differed from that of normal lysates mainly in the lower third of the curve (Figure 3A). The P_{50} of the abnormal lysate was 2.3 mm compared to 2.8 ± 0.2 mm for hemoglobin A.

After addition of 2,3-DPG the result was similar to the one observed after incubation of the red blood cells in the inosine, pyruvate, and phosphate medium. A large shift of the curve was observed above 25% HbO_2. The lower part of the curve was also shifted but to a lesser extent.

When plotted according to Hill's equation, a biphasic aspect of the curve was observed and became more obvious after addition of 2,3-DPG (Figure 3A). The slopes of the upper part of the curves were identical under the different conditions examined and equaled about 2.5. In the lower part of the curve its value was only 1.5. Between these two parts of the curve, an intermediary segment with a slope lower than one was observed. In the presence of 0.1 M NaCl the same biphasic nature was clear (Figure 3B), the shift of the upper part of the curve was much more pronounced after addition of IHP than for 2,3-DPG but the first segment seemed to remain unmodified. A similar phenomenon was observed when the pH of the buffer was decreased.

Components Isolated by Isoelectric Focusing. The amount of hemoglobin Casper which could be recovered from the lysate decreased gradually with storage due to oxidation to methemoglobin and precipitation. Pure hemoglobin Casper could be isolated by electrofocusing from hemoglobin A and from the methemoglobin forms which focused

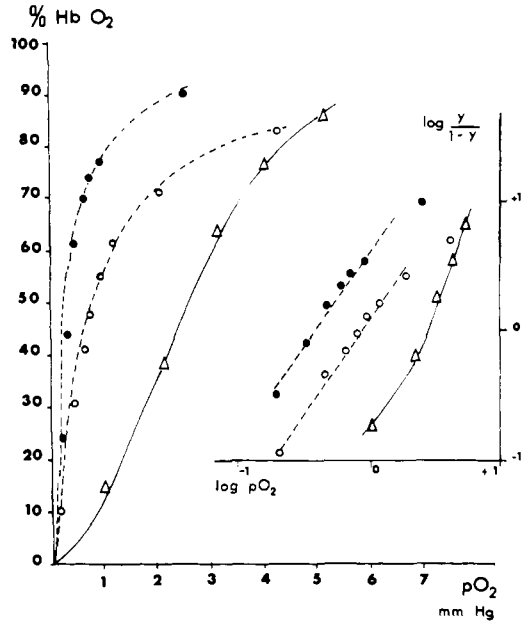

FIGURE 4: Oxygen equilibrium of the components isolated by isoelectric focusing. The measurements were done in 0.05 M bis-tris (pH 7.15) at 25°C. Hemoglobin concentration was 2×10^{-5} M. (●—●) hemoglobin Casper; (O- -O) hemoglobin Casper after addition of 2,3-DPG to a final concentration of 116 μM; (△—△) hemoglobin A.

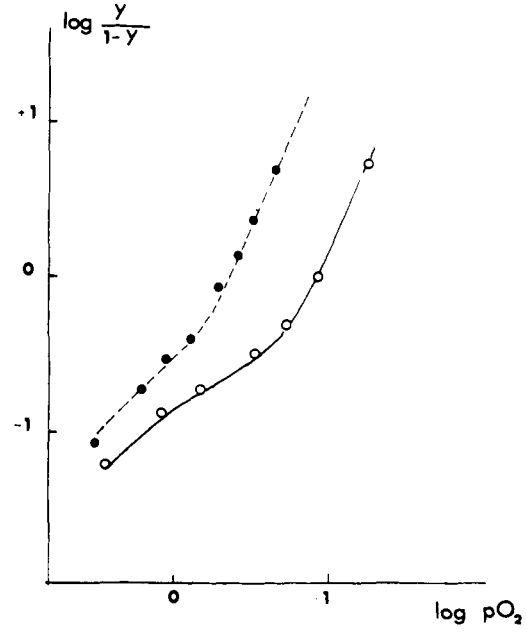

FIGURE 5: Comparison between experimental points and computed curves for a mixture of 80% hemoglobin A and 20% hemoglobin Casper. Stripped hemoglobins: (●) experimental points; (- - -) computed curve assuming for hemoglobin A, P_{50} = 2.8 mm and n = 2.7; and for hemoglobin Casper, P_{50} = 0.36 mm and n = 1.5. After addition of 2,3-DPG to final concentration of 50 μM: (O) experimental points; (—) computed curve assuming for hemoglobin A, P_{50} = 10 mm and n = 2.7; and for hemoglobin Casper, P_{50} = 0.80 mm and n = 1.5.

at higher pH_i. The relative amount of hemoglobin Casper was 30% on the first day after preparing the lysate and decreased to 20% after 1-week storage at 4°C in the oxyhemoglobin form. The decrease of percentage of Hb Casper was due to methemoglobin formation and precipitation. There was no evidence for hemichrome formation. Although some oxidation occurred during the elution of fractions from the electrofocusing gel, the final ratio was less than 5% methemoglobin in all the fractions used for oxygen equilibrium studies.

When hemoglobin A and hemoglobin Casper were eluted from a gel together, the oxygen affinity curve was very similar to the one corresponding to the stripped total lysate. The P_{50} was also 2.3 mm. Pure hemoglobin A prepared in this way had a normal P_{50} value of 2.8 mm. On the other hand, the P_{50} of pure hemoglobin Casper was 0.36 mm and the Hill interaction coefficient was 1.5 (Figure 4).

When a theoretical oxygen equilibrium curve is calculated on an additive basis using the method of Bunn et al. (1972) for a mixture of 80% hemoglobin A and 20% hemoglobin Casper, the computed curve was identical with the curve of the mixture of hemoglobin A and Casper eluted together in the presence and absence of 2,3-DPG (Figure 5).

In order to obtain hemoglobin Casper at least 95% pure, only the center of the abnormal band was cut and eluted. This resulted in a recovery of about 3 mg from 30 mg of hemolysate. Because of the relatively low yield and the small amount of sample available, the action of 2,3-DPG and the effect of pH were assayed at only two points.

After addition of 0.5×10^{-4} M 2,3-DPG the P_{50} of hemoglobin A increased from 2.8 to 7.9 mm. Less of a change in the ratio of P_{50} values of hemoglobin Casper (0.36–0.8 mm) was observed after the addition of saturating amount of 2,3-DPG (1.6×10^{-4} M).

Changes of the P_{50} of hemoglobin Casper with pH were small compared to hemoglobin A. The values obtained for the log P_{50} of hemoglobin Casper at pH 6.5 and 7.5 were −0.15 and −0.40, respectively, as compared to 1.07 and 0.61 for hemoglobin A under the same conditions.

Discussion

In the previous studies of hemoglobin Casper (Jones et al., 1972; Koler et al., 1973) the quantitation of the abnormal hemoglobin was imprecise. The estimates ranged from 20 to 40%. In the present investigation more precise quantitation was achieved by two independent methods. Estimation by the heat stability test (Wajcman et al., 1973) was 30 ± 2% which agreed well with 30% found by densitometer scanning of the gels from electrofocusing when fresh hemolysates were used. Because of its instability, the relative amount of hemoglobin Casper decreased in hemolysates during storage in the cold room.

Although hemoglobin Casper was not resolved from hemoglobin A by chromatography or electrophoresis (Jones et al., 1972), it was possible to separate the abnormal component from hemoglobin A and methemoglobin forms of the two hemoglobins by electrofocusing. The abnormal component thus obtained was between 90 and 100% pure and was satisfactory for functional studies.

The P_{50} values obtained for the abnormal cells suspended in phosphate buffer were lower than normal and consistent with the studies obtained earlier on whole blood (Koler et al., 1973). The more detailed measurement of the oxygen equilibrium data obtained in the present study revealed an abnormal biphasic shape especially in the lower third. Although increasing concentrations of 2,3-DPG caused a shift of the curve to the right in the upper 70%, no change was apparent in the lower 25% of the oxygen affinity curve of whole cells. It was not possible to conclude from these studies of whole cells whether the lack of change in the lower portion of the curve was due to a lack of response of hemo-

globin Casper to 2,3-DPG or was due to the limitations of the measurements. In order to determine if there is an effect of 2,3-DPG on the abnormal component and the possibility of interaction between hemoglobin Casper and hemoglobin A it was necessary to measure the oxygen equilibrium properties of lysate and purified fractions.

The oxygen equilibrium curve of stripped lysate was only slightly different from that of normal hemoglobin when measured in 0.05 *M* bis-tris buffer at pH 7.1. This difference was mainly in the lower third of the curve. A marked difference was observed upon addition of sodium chloride, 2,3-DPG, or IHP as shown in Figure 3A and B. Although the upper portion of the curves shifted very significantly and differently according to the concentrations and kind of ion added, the lower 20% of the curve appeared to shift much less.

The reason for the biphasic nature of the oxygen equilibrium curve of abnormal cells and whole lysate appears to be due to the abnormal oxygen binding properties of hemoglobin Casper. This is apparent from the curve for hemoglobin Casper in Figure 4 which indicates a high oxygen affinity (P_{50} = 0.36 mm) and a low degree of cooperativity. The addition of a saturating concentration of 2,3-DPG shifted the abnormal curve but somewhat less than that observed for normal. The shift is most easily observed in the upper part of the curve but was also detected in the lower part of the curve on linear coordinates of percent oxyhemoglobin vs. P_{O_2}. This small change in the lower part of the curve of hemoglobin Casper being so slight explains why it was not detected in measurements of the abnormal cells. However, it is obvious throughout the whole range from the Hill plot of the data.

In Figure 5 a comparison is made between the oxygen equilibrium curve observed for a mixture of hemoglobin A and hemoglobin Casper and a theoretical curve computed assuming additive effects of hemoglobin A and hemoglobin Casper. The experimental results in the presence and absence of 2,3-DPG are observed to be identical with the computed results. Similar comparisons and results have been obtained by Bunn et al. (1972) for hemoglobin Bethesda. Because of the biphasic nature of the observed curve and its identity with the computed curve it can be concluded that there is no interaction between the hemoglobin Casper and hemoglobin A in the cells or lysate. If hybrid molecules containing one normal β chain and one abnormal β chain exist, a different equilibrium curve would be expected as illustrated by Bunn et al. (1972) for hemoglobin Bethesda and by Imai (1968) for hemoglobin Hiroshima.

The precise relationship between the amino acid substitution in hemoglobin Casper and its high oxygen affinity and lowered cooperativity is difficult to propose because of the multiple changes which a proline substitution in the G helix should make in the tertiary structure of the molecule. The normal contacts which the β G8 residue makes with the heme group are undoubtedly altered in hemoglobin Casper which could account for some of the change in oxygen affinity and the increased rate of oxidation to methemoglobin. However, one might predict that disruption of the G helix by the presence of proline at residue G8 would greatly alter the contacts made in this region with the normal α chain. The fact that this hemoglobin has a different isoelectric point from normal as demonstrated by isoelectric focusing in spite of a neutral substitution of proline for leucine would indicate changes in the tertiary or quaternary structure that alter the exposure of charged groups compared to normal. Alterations in the $\alpha_1\beta_2$ contact sites as probably occur in hemoglobin Casper have been observed to decrease the cooperativity and increase the oxygen affinity in other hemoglobins like hemoglobin Yakima (Jones et al., 1967) and hemoglobin Kempsey (Reed et al., 1968). Although one can predict that these structural changes may shift the equilibrium from the T toward the R state, it is difficult to define more precisely what the changes are without structural analysis by X-ray diffraction methods.

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