Hb Bruxelles, Deletion of Phe\textsuperscript{\beta42}, Shows a Low Oxygen Affinity and Low Cooperativity of Ligand Binding*‡

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Functional studies of partially purified hemoglobin (Hb) Bruxelles, Phe\textsuperscript{\beta42} (CD1) → 0 indicate a major shift in the allosteric equilibrium toward the deoxy (T state) conformation. While Hb A shows a roughly symmetrical oxygenation curve with maximum cooperativity near half-saturation, Hb Bruxelles shows mainly properties of the low affinity (T state) form. The oxygen equilibrium curves for purified (\textgtrsim80%) Hb Bruxelles show little cooperativity and a \textit{P}_\text{50} (without 2,3-diphosphoglycerate) about twice that of Hb A. The low cooperativity for Hb Bruxelles is partially compensated by an increase in oxygen affinity of the deoxy conformation and a lower 2,3-diphosphoglycerate effect.

The \textit{ \beta} chains of normal Hb have consecutive phenylalanine residues at positions 41 and 42. DNA sequencing studies of Hb Bruxelles showed a deletion of the codon TTT, which corresponds to residue Phe\textsuperscript{42}.

The \textit{CO} rebinding kinetics after flash photolysis show mainly the slow phase, characteristic of \textit{CO} binding to the deoxy conformation. In phosphate buffer at pH 7, the slow phase dominates even at low photolysis levels, where the main reaction is ligand binding to the triply liganded form. This indicates a switchover point, from the deoxy to oxy conformation, occurring beyond three ligands for Hb Bruxelles. There are few natural mutants that show a change in the oxygen affinity and cooperativity as large as that observed for Hb Bruxelles.

It has been known for many years that mutations of the hemoglobin (Hb) \textit{ \beta} chains in the vicinity of the heme group and at the CD corner of the subunits are responsible for more or less severe instability, hemolytic anemias, and an accelerated rate of autooxidation (1). Specifically, position \textit{ \beta}42 (CD1) Phe is one of the most highly conserved residues.

In all cases that have been described, the functional studies have been performed in erythrocyte suspensions or heterologous hemolysates because the abnormal component could not be separated and/or was too unstable to prevent precipitation or oxidation during the purification procedure. Whole blood studies have revealed that these hemoglobins (Phe\textsuperscript{41} → Tyr, Phe\textsuperscript{41} → Ser, Phe\textsuperscript{42} → Leu, Phe\textsuperscript{42} → Val, or Phe\textsuperscript{42} → Ser), while expressing varying clinical courses and Bohr effects, display a low oxygen affinity, low cooperativity in ligand binding, and normal or close to normal interactions with organophosphate effectors (2–14).

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1 The abbreviations used are: HPLC, high pressure liquid chromatography; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; metHb, methemoglobin; cyanometHb, cyano-methemoglobin; 2,3-DPG, 2,3-diphosphoglycerate.

In 1989, our group discovered a new hemoglobin variant in a 4-year-old girl who exhibited a severe chronic hemolytic anemia and cyanosis. During the first 5 years of her life she had several hyperhemolytic events, which required in one case a transfusion. Her blood hemoglobin concentration is about 10 g/dl, indicating that her anemia has stabilized. Reverse phase HPLC\textsuperscript{1} of the abnormal \textit{ \beta} chains revealed the presence of a fast moving \textit{ \beta}5-5 peptide containing two phenylalanine residues instead of three in normal \textit{ \beta} chains (at positions 41, 42, and 45). Sequence analysis showed that either the \textit{ \beta}41 or \textit{ \beta}42 residue was missing (15).

The aim of this paper is to report on the functional properties of isolated Hb Bruxelles obtained after a preparative isoelectric focusing. As the oxygen binding properties of this Hb variant were greatly shifted toward the low affinity conformation, kinetics of \textit{CO} rebinding after flash photolysis were also carried out to study the properties of the binding of the fourth ligand.

MATERIALS AND METHODS

Purification of Human Adult Hb A—Hemolysate was prepared from fresh blood by the routine method (16). The purity and the quality of the sample was checked by analytical isoelectric focusing and by absorbance spectra of the oxy and the carbonmonoxym forms (250–700 nm).

Preparation of Hemoglobin Bruxelles—The total hemolysate was prepared as for Hb A. Hb Bruxelles was partially purified by two successive preparative isoelectric focusing assays (on a granulated bed with amphotoline, \textit{pH} 6–8) of this hemolysate flushed under 1 atm of \textit{CO}. The fraction corresponding to hemoglobin Bruxelles (about 80% of the total) was collected and then eluted and concentrated by ultrafiltration (Amicon) and finally equilibrated under 1 atm of \textit{CO}.

Hb Bruxelles has an isoelectric point (\textit{pI} = 7.04) located between that of Hb A (\textit{pI} = 6.98) and Hb F (\textit{pI} = 7.10). An oxyhemoglobin solution was obtained from the stock carboxy form by equilibration under 1 atm of oxygen at 4 °C under intense light to aid CO removal. The absorbance spectra of the oxy and carboxy forms (250–700 nm) were normal.

Oxygen Equilibrium Curves—Oxygen equilibrium curves were measured by a continuous method using a Hemox analyzer, as described previously (17). Experimental conditions for red blood cell suspensions were 50 mM bis-Tris buffer, 140 mM NaCl at 37 °C, \textit{pH} 7.4. For experiments on striped hemoglobin solutions (60 mM in heme) purified by isoelectric focusing assays, we used 50 mM bis-Tris buffer at \textit{pH} 7.2, 100 mM NaCl at 25 °C with 50 mM EDTA and 20 mM catalase added. Due to the higher autooxidation rate of Hb Bruxelles relative to Hb A, 10% percent of metHb was present at the end of the oxygen equilibrium studies.

Autoxidation—Autoxidation studies were made using oxyhemoglobin solutions equilibrated under 1 atm of oxygen at 37 °C in 20 mM phosphate buffer at \textit{pH} 7.0 (\textit{heme} \textgtrsim 30 mM). Absorbance spectra were recorded initially every 5 and then every 15 min for 6 h using an SLM-Amino (DW 2000) spectrophotometer.

Estimation of the Percentage of Ferrous Forms—The absorbance...
spectra of oxyhemoglobin and methemoglobin are distinct in the visible region (350–700 nm). We used this property to follow the autoxidation kinetics of oxyhemoglobin to methemoglobin. The ferrous hemoglobin percentage present at a given time was determined by fitting the absorbance spectrum as a fraction of the initial spectrum of the fully oxygenated hemoglobin and that of the fully completely oxidized hemoglobin, determined after the addition of an excess of potassium ferri cyanide. At pH 7.0, the percentage of ferrous hemoglobin A varies according to a monoexponential law (18).

Flash Photolysis Kinetics—The bimolecular kinetics after photodissociation of CO provide a sensitive test for Hb function. The recombination rates for the two conformations (R and T) differ by about a factor of 30, accounting for most of the factor of 100 difference in ligand affinity. The kinetics are thus biphasic, reflecting the two allosteric forms, with the slow rate corresponding to rebinding to the deoxy (T state) form. Furthermore, one can vary the photolysis energy to change the fraction dissociation: at low energies the main photoproduct is triply liganded tetramers, which normally remain in the rapid (R) state, while high dissociation levels produce more deoxy and single liganded tetramers, which switch to the low affinity conformation. Since the R to T transition requires about 100 μs, similar to the rate for CO rebinding to the R state (at 1 mM CO), variation of the CO concentration is also a useful experimental parameter to detect the transition.

Hb A/Hb Bruxelles Hybrids—In order to determine whether Hb A and Hb Bruxelles readily exchange dimers, experiments were made with mixtures of the two parent forms. After photolysis of the purified Hb Bruxelles-CO samples, Hb A-CO or methHb A-CN were added to study the effect on the CO rebinding kinetics. An equal mixture was used when both samples were CO, with a small excess of dithionite to serve for oxygen binding equilibrium experiments. Even at 37 °C, the autoxidation rate of purified Hb Bruxelles sample shows little cooperativity, n = 1.1, and a 2-fold decrease of the oxygen affinity (Table I), with a half-saturation at 9.8 mm Hg compared with 5.1 mm Hg for purified Hb A (at pH 7.2, 100 mM NaCl, 25 °C). The 2,3-DPG effect for Hb Bruxelles was decreased to a large extent relative to Hb A (Table I). At the end of the oxygen binding experiments, a percentage of about 10% of methemoglobin was measured for Hb Bruxelles.

Absorbance Spectra

Absorbance spectra of hemoglobin Bruxelles oxyform and carbamonyox form were recorded between 250 and 700 nm. The ratio between the maximum of absorbance in the Soret and UV analyses gave for the oxy form a value of 3.8 (normal = 4) and for the carbamonyox form a value of 5 (normal = 5), indicating a normal heme binding (data not shown), although a small percentage of metHb was detected in those samples.

Oxygen Equilibrium Curves

Red Blood Cells—The value of P50 at pH 7.4 measured in red blood cells containing the natural mixture of Hb A and Hb Bruxelles is 1.5-fold higher than that measured in normal red blood cells (Table I). These results are in agreement with those measured in 1989 (15).

Purified Hemoglobin Solution—Oxygen equilibrium curves of purified hemoglobin solutions are shown in Fig. 2. The purified Hb Bruxelles sample shows little cooperativity, nmax = 4, and a 2-fold decrease of the oxygen affinity (Table I), with a half-saturation at 9.8 mm Hg compared with 5.1 mm Hg for purified Hb A (at pH 7.2, 100 mM NaCl, 25 °C). The 2,3-DPG effect for Hb Bruxelles was decreased to a large extent relative to Hb A (Table I). At the end of the oxygen binding experiments, a percentage of about 10% of methemoglobin was measured for Hb Bruxelles.

Autoxidation

Hb Bruxelles showed a 2-fold increase in the autoxidation rate relative to Hb A. This is slightly more than expected from the correlation between a lower oxygen affinity and accelerated oxidation as reported in the case of myoglobin (20). The fact that Hb Bruxelles is not fully saturated (under 1 atm of oxygen at 37 °C) also contributes to the accelerated autoxidation rate. The final metHb spectrum (Fig. 3) was slightly different from metHb A, resembling that of metHb A at a slightly higher pH value.

Flash Photolysis Kinetics

Rebinding kinetics for Hb A and Hb Bruxelles in phosphate buffer are shown in Fig. 4. The curves for each Hb correspond to three levels of photodissociation. Hb A shows a large variation of the slow fraction with laser energy, while Hb Bruxelles is less affected, in agreement with the low cooperativity observed for oxygen binding equilibrium experiments. Even at
low photodissociation levels, where triply liganded tetramers are the main photoproduct, the high fraction of slow phase persists (Fig. 4). This indicates that the low affinity (T state) conformation is favored even with three ligands bound to Hb Bruxelles.

The equilibrium and kinetic data both indicate that the allosteric equilibrium for Hb Bruxelles is greatly shifted to the low affinity conformation. There is little difference in simulations of equilibrium data for a switchover point (50% of each conformation) of 3.5 or higher. It is therefore difficult to determine the fraction T state for the fully liganded form. Alternate tests or probes are required. One such probe is the fraction geminate phase in the flash photolysis kinetics. It corresponds to the fraction of CO photodissociated that recombines directly to the heme without escaping from the protein. Previous results indicate that the fraction geminate is much lower for T state Hb (21–22), since its rebinding rate is much lower than for liganded (or R state) Hb. The bimolecular yield at 25°C was normal for Hb Bruxelles (about 53%, as compared with 48% for Hb A), indicating that the fully liganded Hb-(CO)_4 is mainly in the R state conformation.

The bimolecular signal increased with temperature, as for Hb A, but then dropped near 68°C (70°C for Hb A), indicating the onset of thermal denaturation. Also, the fraction of slow bimolecular kinetics was lower for samples equilibrated under 1 atm of CO than for those under 0.1 atm of CO (data not shown). This indicates that the R to T transition occurs after photodissociation and that the rate is competitive with rebinding to Hb in the R state (which requires about 0.2 ms under 1 atm of CO).

**Hybrids**

The addition of Hb A-CO to Hb-Bruxelles-CO samples led to an intermediate result (data not shown). The kinetics were only slightly different from a simple sum of the kinetics for the two parent species; it is therefore difficult to quantify the actual amount of hybrid form and its properties. The formation of such hybrids may show deviations from a random distribution (23).

We also prepared mixtures of ferrous Hb Bruxelles-CO and cyan-metHb A. These results were easier to interpret, since the CN ligand does not have an interfering signal. The kinetics showed a net decrease in the slow fraction after the addition of cyan-metHb A. The slow fraction (at low photolysis level) decreased by half at a ratio of Hb A to Hb Bruxelles of about 4:1 (Fig. 5). Control experiments with addition of cyano-metHb A to Hb A-CO showed less slow phase than the hybrids with Hb Bruxelles; this indicates that the hybrid (with a single mutated β chain) maintains a shift toward the T state relative to Hb A.
The spatial configuration (4, 5). Low oxygen affinity is also transcribed for the shift toward the low affinity state (2). Thus, the substitution of tyrosine for phenylalanine causes little distortion of the protein stability than Phe41 (C7), since mutations of position 41 such as substitutions at served residues, appears to be more involved in the protein structure. This region of the exon appears to be a hot spot for deletion unambiguously, since the Phe codon in position 41 is TTC (Fig. 1).

The oxygen affinity is decreased in all the variants substituted at position 41 (22), for example, 75% photodissociation; *, 8% photodissociation) of the purified Hb Bruxelles, cyan-metHb A was added to obtain different ratios of the two types of Hb. The change in kinetics indicates the formation of a hybrid species through dimer exchange. Experimental conditions were as follows: 50 mM bis-Tris buffer at pH 7.2, 100 mM NaCl, 25 °C, 0.1 atm of CO.

**DISCUSSION**

The mutation responsible for Hb Bruxelles is a deletion of the Phe codon in position 42 (TTT); this assignment is now unambiguous, since the Phe codon in position 41 is TTC (Fig. 1). This region of the exon appears to be a hot spot for deletion or point mutations. Indeed, in a stretch of 16 base pairs around Phe42, four other deletions leading to a frameshift or abnormal hemoglobins and several mutations leading to structural variants have been reported. The mechanism underlying such mutational events is probably sequence-directed. In the region of Phe42, two direct repeats of five base pairs (CTTTG) may be involved in the mechanism of mutagenesis (24) (Fig. 1).

Phenylalanines 41 (C7) and 42 (CD1) are conserved in all normal non-α-globin chains of mammalian hemoglobins and seem to be critical in the structural integrity and function of the hemoglobin molecule (Fig. 6). The other structural variants previously described at these positions are mainly point mutations. Phe42 (CD1), which is one of the three most highly conserved residues, appears to be more involved in the protein stability than Phe41 (C7), since mutations of position 41 such as Hb Mequon (2, 3) or Hb Denver (4, 5) do not lead to great instability. On the contrary, substitutions of Phe42 lead to very unstable variants (such as Hb Hammersmith-Chiba, Hb Bucuresti-Louisville, and Hb Warsaw-Sendagi). Hb Bruxelles (15), like Hb Hammersmith (Phe42 → Ser) (12–14) results in an active hemolytic disease compared with Hb Bucuresti (Leeu) (6, 7) and Hb Sendagi (Val) (8–11) because Ser is uncharged but polar, whereas Leu and Val are uncharged and nonpolar. Hb Niteroi presents a three-amino acid deletion between β43 and β45 (Glu-Ser-Phe) or between β42 and β44 (Phe-Glu-Ser) and, like Hb Hammersmith, is unstable (25).

The oxygen affinity is decreased in all the variants substituted at position 41 or 42 by an amino acid with a much smaller side chain (Hbs Denver, Hammersmith-Chiba, Bucuresti-Louisville, Warsaw-Sendagi) or where one of the phenylalanines is deleted (Hb Bruxelles). On the contrary, the oxygen affinity is only slightly reduced in Hb Mequon (Phe41 → Tyr), presumably because the tyrosine and phenylalanine have side chains of similar size; in this case, an additional hydrogen bond in the deoxy conformation, at the αβ interface, could be responsible for the shift toward the low affinity state (2). Thus, the substitution of tyrosine for phenylalanine causes little distortion of the spatial configuration (4, 5). Low oxygen affinity is also observed for Hb F-Cincinnati Phe41 (C7) → Ser (26).

The consequences could be more severe in the case of Hb Bruxelles, compared with the other point substitutions, because the Phe is deleted rather than replaced by another residue. This will lead to some perturbation of the location (and therefore its contacts) of the residues immediately following the deletion. For example, the glutamic acid, originally in position 43, will now shift to position 42 in the sequence. It is not yet known whether the effects of the deletion will localize in the CD corner.

**Formation of Hybrids**—When a single gene is involved in the expression of a protein, the organism will encounter the full effects of the new properties of the protein. Large changes in physiological properties may go beyond the limits tolerated by the organism. In the case of Hb, an excessively high oxygen affinity would lead to oxygen binding at the lungs but poor release at the tissues; conversely, an extremely low affinity would result in a Hb that does not bind ligands and simply circulates as deoxy-Hb.

The situation is more complicated for Hb possessing an abnormal chain, due to the fact that there are two types of subunits, the α and β chains, and there are four genes for α chains and two for β chains. The abnormal properties of a single abnormal β subunit within the tetrameric (α2β2) Hb may therefore be reduced relative to a tetramer where two subunits are affected. If one gene is affected, leading to the production of 50% mutant for the β chains, a random mixture of subunits would lead to only 25% tetramers with both β chains affected (with 50% hybrid and 25% pure Hb A). Tetramers with only a single mutant β subunit per tetramer may show smaller changes in the physiological properties. Such studies involving one or two affected chains per tetramer have been conducted in the case of the dimer-tetramer equilibrium for Hb (23, 27); these workers observed deviations from the binomial distribution for certain mutants. If the distribution is random, then the hybrid (dimer Hb A/dimer mutant) will be the dominant species; it is thus of interest to determine the relative populations and the properties of the hybrid.
A mixture of two types of Hb can lead to some compensation or dilution effect. The best known case for Hb is sickle cell disease, due to Hb S Glu$^6\rightarrow$Val (1); while patients with a single gene affected show no symptoms, the homozygous cases are severely affected. Hb S tetramers have the same oxygen binding parameters as Hb A tetramers, but at high concentrations the deoxy Hb S tetramers aggregate to form long strands, which distort the cell to various "sickle" forms that can block the capillaries.

In the case of Hb Bruxelles, the addition of cyano-metHb A influenced the CO rebinding kinetics, demonstrating the formation of hybrids through the exchange of dimers. If no exchange occurred, one would expect no change in kinetics, since the cyano-metHb provides no signal. The data for the mixture are compatible with a random distribution, where the hybrids have intermediate properties, although other simulations are possible.

To estimate the oxygen binding properties of a mixture of Hb A and a mutant Hb, one must consider the distribution of tetrameric forms. At rest only about one-fourth of the ligands are delivered. The organism can thus support a fraction of poorly adapted Hb, although the respiration may be limited to nonstrenuous situations. The actual distribution will depend on the fraction of each type of chain. The expression and stability of these chains will also play a role (1). As observed for other mutants, Hb Bruxelles represents less than half (40%) of the non-α chains. This can play a significant role, since the amount of tetramers with two mutant β chains would decrease from 25 to 16%. This calculation assumes a random distribution; perturbation of the dimer-tetramer equilibrium by the mutation will obviously change the percentages; these changes could occur for the deoxy or oxy (or both) conformations. In other cases, the stability may influence the final distribution. For example, mutants that are unstable may form precipitates that can be removed by the spleen (28). The observed fraction of mutant Hb may therefore depend on the age of the cells, and the distribution of tetrameric species will change accordingly.

Separation of Affinity and Allosteric Effects—The dominant effect for the difference between Hb A and Hb Bruxelles is a large shift in the allosteric equilibrium toward the deoxy conformation. The shift is so large that both the oxygen equilibrium and CO kinetic studies show mainly T-like properties, although the T state for Hb Bruxelles is shifted toward a higher affinity. Hb Kansas is another low affinity Hb, although the respiration may be limited to nonstrenuous situations. The actual distribution will depend on the fraction of each type of chain. This can play a significant role, since the fraction of each type of chain. The expression and stability of these chains will also play a role (1). As observed for other mutants, Hb Bruxelles represents less than half (40%) of the non-α chains. This can play a significant role, since the amount of tetramers with two mutant β chains would decrease from 25 to 16%. This calculation assumes a random distribution; perturbation of the dimer-tetramer equilibrium by the mutation will obviously change the percentages; these changes could occur for the deoxy or oxy (or both) conformations. In other cases, the stability may influence the final distribution. For example, mutants that are unstable may form precipitates that can be removed by the spleen (28). The observed fraction of mutant Hb may therefore depend on the age of the cells, and the distribution of tetrameric species will change accordingly.

Conclusion—Hb Bruxelles represents one of the largest deviations in oxygen binding properties ever observed for natural mutants. Only certain cross-linked Hbs (30), synthetic mutants at position Asn$^{102}$ (31), or use of external effectors (32) lead to lower affinities or less cooperativity. The large shift in the allosteric equilibrium is partially compensated by a T state with a higher oxygen affinity and a decreased 2,3-DPG effect. The patient does show anemic symptoms, indicating that Hb Bruxelles may represent a limiting case for the change in functional properties of Hb.

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