A recombinant human hemoglobin with asparagine-102(β) substituted by alanine has a limiting low oxygen affinity, reduced marginally by chloride

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(RECEIVED August 8, 1994; ACCEPTED October 12, 1994)

Abstract

A recombinant (r) mutant hemoglobin (Hb) with Asn-102(β) replaced by an Ala (N102A(β)) has been prepared by PCR amplification of a mutagenic DNA fragment and expression of the recombinant protein in yeast. The side chain of Asn-102(β) is part of an important region of the $\alpha_1\beta_2$ interface that undergoes large structural changes in the transition between the deoxy and oxy conformations. Three natural mutant Hbs with neutral substitutions of Thr, Ser, or Tyr at this site have low oxygen affinities because a hydrogen bond between Asn-102(β) and Asp-94(α) in normal HbA was considered to be absent in these mutants, thereby destabilizing the oxy conformation in favor of the deoxy conformation. This proposal has been tested by expression of an rHb containing alanine at position 102(β); alanine was chosen because its methyl side chain cannot participate in hydrogen bond formation, yet it is small enough not to disrupt the subunit interface. The nature of the desired replacement was established by sequencing the entire mutated β -globin gene as well as the tryptic peptide containing the substitution. Further characterization by SDS-PAGE, isoelectric focusing, HPLC analysis, mass spectrometry, amino acid analysis, and sequencing of the mutant tryptic peptide confirmed the purity of the rHb. Its oxygen binding curve (2.4 mM in heme) in the absence of chloride showed that it had a very low oxygen affinity with a P_{50} of 42 mm Hg. In the presence of added chloride (0.5 M), its oxygen affinity was further reduced only slightly to a P_{50} of 49 mm Hg. In contrast, the oxygen affinity of HbA was lowered two- to threefold by the same concentration of chloride. Comparison of the properties of the rHb N102A(β) with those of the rHb with Asp-99(β), which is at this same subunit interface, substituted by a Lys (Yanase H, et al., 1994, *Protein Sci* 3:1213–1223), demonstrates how the judicious choice of the amino acid substitution based upon the properties of natural mutations at a particular site can further enhance our understanding of the role of certain amino acid side chains in Hb function.

Keywords: chloride effect; hemoglobin; low oxygen affinity; mutagenesis

Hemoglobin (Hb) Kansas is a naturally occurring mutant Hb in which asparagine at position 102 of the β -chain is replaced by a threonine residue (Bonaventura & Riggs, 1968; Ogawa et al., 1972; Gibson, 1973; Atha & Riggs, 1976; Atha et al., 1979). This mutation occurs in an important region of the $\alpha_1\beta_2$ interface, which undergoes large rearrangements in the transition between the oxy and deoxy conformations of Hb (Perutz & TenEyck, 1971; Perutz et al., 1976; Perutz, 1990) (Fig. 1). It

has been proposed that Hb Kansas has a low oxygen affinity because the hydrogen bond that usually occurs between Asn-102(β) and Asp-94(α) in normal Hb to stabilize the oxy conformation is absent in this mutant Hb, thus shifting the equilibrium toward the deoxy conformation (Greer, 1971). Two other natural mutants with neutral substitutions at this position and also with low oxygen affinities are Hb Beth Israel (Asn-102(β) $\rightarrow$ Ser) (Nagel et al., 1976) and Hb St. Mande (Asn-102(β) $\rightarrow$ Tyr) (Arous et al., 1981; Poyart et al., 1990). However, in these three natural mutant Hbs, each with hydroxyl-bearing substitutions, hydrogen bonding remains a possibility and indeed has been found for Hb St. Mande (Poyart et al., 1990). Therefore, the question arose

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Fig. 1. Segment of the $\alpha_1\beta_2$ interface. From Perutz and Ten Eyck (1971).

whether there were alternative mechanisms for the low oxygen affinity of these mutants. Hence, in this communication, we report the effects of the substitution of an Ala residue at position 102(β) on the functional properties of Hb. We also compare its drastically different properties with those of a recombinant (r) Hb in which Asp-99(β) at an adjacent site in the same $\alpha_1\beta_2$ interface was substituted by a Lys (Yanase et al., 1994) (Fig. 1).

A fourth natural mutant at position 102, Hb Richmond, has a Lys instead of an Asn residue at this site (Efremov et al., 1969). Winslow and Charache (1975) found that the oxygen affinity of Hb Richmond was higher at low pH and lower at high pH compared to HbA, with a crossover point near physiological pH. According to Greer (1971), Lys-102(β) in Hb Richmond can form a salt bridge with Asp-94(α) in the oxy conformation.

To express rHb, we employ a yeast plasmid that contains both human α - and β -globin genes on the same plasmid (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b). This expression system uses endogenous yeast heme to produce a soluble Hb tetramer that is processed at its N-terminus and has the same conformation and functional properties as natural human Hb isolated from human red cells, as demonstrated by a variety of techniques (Martin de Llano et al., 1993a, 1993b). In addition, it has recently been shown that two rHb expressed in this system have the same CD spectra as natural Hb from 200 to 650 nm, a range that covers both overall protein conformation and the interaction with heme (Martin de Llano & Manning, 1994). Thus, this yeast expression system is ideal to study the effects of specific changes at important regions of either the α - or β -chains in order to gain a fuller appreciation of the details of Hb function.

Results

Identification of the mutation on the β -globin gene

Using PCR-assisted mutagenesis, we prepared mutants to express various substitutions at position 102 of the β -chain of Hb. The recombinant plasmids pBSK-BETA^(M), which were constructed to insert the amplified *Bam* H1 fragment with the mutagenic A or B mix primer, were identified by DNA sequencing as having a mutated base at position 102. Eleven β -globin mutants were isolated—N102A, N102L, N102R, N102S, N102F, N102D, N102E, N102H, N102P, N102M, and the double mutant N102W/F103I. Of these mutant β -globin genes, pBSK-BETA^{N102A} was selected because of the small size and neutral nature of its side chain, and the excised mutant β -globin gene was subcloned into pGS389 to construct pGS389 N102A(β). Plasmid pGS389 N102A(β) was transformed into the *Saccharomyces cerevisiae* GSY112 strain and the rHb N102(β) was expressed in the yeast system by the procedures described previously (Martin de Llano et al., 1993a, 1993b).

Purification of the N102A(β) rHb mutant

The growth of the yeast was not affected by the expression of this rHb mutant as previously found for other Hbs expressed in this system (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). The rHb, which was purified first by batchwise chromatography on CM-52 and then by HPLC as described above, eluted as a single, symmetrical peak on the Synchropak CM 300 column (Martin de Llano et al., 1993a).

Isoelectric focusing

Analysis of the purified recombinant Hb by isoelectric focusing between pH 6 and 8 showed that it migrated as a single tight band in a position slightly cathodal to that of HbA (Fig. 2). This difference in pI was unexpected considering the neutral nature of the substitution of an Ala for an Asn, but anomalous chromatographic behavior of Hb Kansas and of Hb St. Mande have been noted previously by Bonaventura and Riggs (1968) and by Poyart et al. (1990), respectively.

Fig. 2. Isoelectric focusing of N102A(β) mutant Hb. A gel (pH 6–8) (Isolab) was electrophoresed at 10 W for 40 min and stained with 0.2% bromophenol blue. Lane a: N102A(β) mutant Hb; lane b: a natural Hb.

Separation of α - and β -chains

The purified recombinant N102A(β) Hb was subjected to HPLC analysis under denaturing conditions in order to separate and analyze the α - and β -globin chains. The mutant β -chain eluted at 28.5 min, significantly later than the β -chain from natural HbA (25.0 min) (Fig. 3). This result is consistent with the more hydrophobic nature of the mutant peptide where Ala is substituted for Asn. The α -chains from the recombinant mutant and the normal Hb eluted in the same positions within experimental error (31.2 min and 32.0 min, respectively), consistent with the absence of a substitution in the α -chain. Based on the significant differences in the values for serine, glutamic acid, glycine, alanine, and valine between the α - and β -chains, the results of the amino acid analysis permitted their assignments (data not shown).

Peptide mapping

The mutant β -chain obtained as described in Figure 3 was reduced and carboxymethylated as described by Crestfield et al. (1963) and then digested with trypsin, as described previously (Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). The digest was applied to a Vydac C-18 column and eluted with the gradient described below. Comparison of the pattern of the tryptic peptide map of the mutant β -globin chain with the corresponding tryptic peptide map of the normal β -globin chain showed one major peak present in the mutant β -chain (Fig. 4, open arrow) that was absent in the tryptic peptide map of normal β -globin, which itself had a peptide (Fig. 4, closed arrow) that was absent in the mutant Hb. The late elution of the mutant peptide compared with the corresponding peptide from normal Hb was consistent with its increased hydrophobicity. The remainder of the peptide map (not shown) was identical for the two β -globin chains.

Fig. 3. Separation of α - and β -globin chains of N102A(β) mutant and of natural HbA. The mutant and natural Hb were purified by HPLC and then chromatographed on a Vydac C-4 column as described in the text. **A:** Elution profile of N102A(β) mutant (100 μ g). **B:** Elution profile of natural Hb (115 μ g).

Fig. 4. Tryptic peptide maps of β -chain of N102A(β) mutant and a natural Hb. The β -chains of each Hb were isolated on a Vydac C-4 column as shown in Figure 3, then reduced, carboxymethylated, and digested with *N*-tosyl-L-phenylalanine chloromethyl-trypsin, and chromatographed on a Vydac C-18 column as described in the text. The closed and open arrows indicate unique peaks in digests of natural and mutant β -globin chains, respectively. The peptide in the upper peak (open arrow) from mutant Hb was hydrolyzed and then subjected to protein sequencing.

Sequence of the mutant peptide

The unique tryptic peptide isolated from the mutant β -chain, which was purified with a yield of 32.3% as described above, was subjected to amino acid sequencing. The results showed that this peptide comprised amino acid residues 83–104 of the β -chain and Ala was found in high yield at position 102 (Fig. 5), confirming the nature of the substitution. In this peptide, trypsin did not cleave the peptide bond after Lys-95(β) because of the presence of Asp at position 94.

Mass spectrometric analysis

The purified native recombinant N102A(β) tetramer was subjected to mass spectrometric analysis as described in the Materials and methods. The results (Table 1) indicated that the mass of the recombinant β -chain was consistent with the calculated value of 15,825, which is 43 mass units lower than a normal β -chain, exactly that calculated for the replacement of an Asn by an Ala. The mass of the α -chain was 15,126, which is the same as the mass of the normal α -chain from human HbA.

Fig. 5. Amino acid sequence of mutant tryptic peptide from N102A(β) mutant Hb. The tryptic peptide that was sequenced was isolated as described in Figure 4. The numbers under the sequence are the repetitive yields at each cycle of Edman degradation. Cys-93 was present as the S-carboxymethyl derivative after treatment with iodoacetate as described in the text. The underlined Ala at position 102 is the site of the mutation.

Spectral properties of recombinant N102A(β) mutant Hb

The spectrum of the mutant Hb in the ultraviolet and visible range indicated that the ratios of the peak absorbances were practically identical to those of normal HbA (data not shown).

Tetramer-dimer dissociation

The extent of dimer formation of the mutant Hb was compared to that of normal HbA by two procedures, i.e., filtration of the haptoglobin-Hb dimer complex (Table 2) and quenching of haptoglobin fluorescence upon binding of Hb dimers (Fig. 6). The results found by these two techniques were in agreement and indicated that in the micromolar concentration range, the N102A(β) mutant in both the oxy and deoxy conformations behaved in a manner practically identical to that of HbA. The small amounts of dimer found for both the normal and recombinant mutant Hb under deoxygenated conditions were likely due to the slow displacement of the tetramer-dimer equilibrium toward dimer formation induced by the presence of haptoglobin.

Oxygen binding properties

A P_{50} value of 42 mm Hg was found for the recombinant mutant N102A(β) Hb (Fig. 7; Table 3). Because oxygenation rate was slow for this low-affinity mutant, we changed the usual gas mixture containing 25% O₂ to one containing 50% O₂. It is especially important to vary this parameter with low-affinity mutants. Another consideration in obtaining accurate values is the actual extent of oxygen saturation attained. For example, for a particular turtle Hb with a low oxygen affinity, Lapennas et al. (1981) calculated that the observed P_{50} value differed from the

true value by as much as 5 mm Hg, depending on the value taken for 100% saturation. In our studies comparing the effects of 25% O₂ with 50% O₂, we found that, although P_{50} values were lower with the latter gas mixture, the degree of response to added chloride did not change significantly (Table 3), i.e., the oxygen affinity of HbA was lowered more than twofold by addition of chloride, but the oxygen affinity of the N102A(β) mutant Hb was decreased by only 10–20% and these values did not vary with either 25% O₂ or 50% O₂. The results suggest that the major contribution to the lowering of the oxygen affinity was due to the substitution itself and not to added chloride. It is important to point out that other recombinant mutant Hb expressed in this same yeast system, i.e., HbS, interacted normally with chloride in terms of a lowered oxygen affinity (Martin de Llano et al., 1993a, 1993b).

Discussion

The extremely low oxygen affinity of the recombinant N102A(β) mutant Hb is consistent with the general trend reported for the natural mutants with hydroxyl-containing neutral substitutions at this position, i.e., Hb Kansas (Asn $\rightarrow$ Thr), Hb Beth Israel (Asn $\rightarrow$ Ser), and Hb St. Mande (Asn $\rightarrow$ Tyr). However, the magnitude of the lowering of the oxygen affinity of the recombinant mutant Hb N102A(β) was significantly greater than that of any of these three natural mutants. The results suggest a nearly complete shift in the deoxy-oxy equilibrium toward the deoxy conformation due to the lack of any interaction between the side

Table 1. Mass spectrometric analysis of the N102A(β) mutant Hb

	Mass
α -Globin	
Measured	15,126.8
Calculated	15,126.4
β -Globin	
Measured	15,826.1
Calculated	15,825.2

Table 2. Extent of dimer formation of HbA and the N102A(β) recombinant mutant as determined by haptoglobin binding^a

	% Dimer
Oxy HbA	74
Oxy N102A(β)	67
Deoxy HbA	14
Deoxy N102A(β)	14

^a The Hb concentrations were 0.5 μ M as tetramer and the haptoglobin concentrations were 1.0 μ M. The mixing and the filtration with the Centricon apparatus were performed as described previously (Yanase et al., 1994). When present, the sodium dithionite concentration was 0.6 M.

Fig. 6. Quenching of haptoglobin fluorescence by Hb dimers. The quenching of haptoglobin fluorescence by binding of Hb dimers under oxygenated conditions was determined as described in the text for various ratios of Hb to haptoglobin shown in the figure.

chains of Ala-102(β) and Asp-94(α). This argument implies that some hydrogen bond interaction still exists between the β -hydroxyl groups of the neutral natural mutants and Asp-94(α) in the oxy conformation. Indeed, in Hb St. Mande, Poyart et al. (1990) found evidence for a hydrogen bond between the phenolic group of Tyr-102(β) and Asp-94(α) in the oxy conformation, and also involving Tyr-42(α) in the deoxy conformation.

The very modest further reduction in oxygen affinity upon addition of chloride to the recombinant N102A(β) mutant Hb suggests that the limiting oxygen affinity may have already been nearly reached even in the absence of allosteric regulators. In contrast, Hb St. Mande still responds to chloride, but with a reduced efficiency compared to HbA (Poyart et al., 1990).

The natural mutant Hb with substitutions at position 102(β) have an increased tendency to dissociate to dimers in their oxygenated states (Bonaventura & Riggs, 1968; Ogawa et al., 1972; Gibson, 1973; Atha & Riggs, 1976; Atha et al., 1979). Thus, the dissociation constant of Hb Kansas in its oxygenated state is in the millimolar range, about 50 times greater than that for oxy HbA, but in its deoxygenated state, Hb Kansas dissociates to the same extent as normal HbA. This property plays a role in the determination of its functional properties. For example, an important difference in the studies with Hb Kansas and with the N102A(β) recombinant mutant is the Hb concentrations used for measurement of their oxygen binding curves. In the present study, relatively concentrated solutions (millimolar range in

Fig. 7. Oxygen-binding curves of N102A(β) mutant and natural HbA. The oxygen equilibrium curves were determined at 37 °C on Hb (2.4 mM in heme) in 50 mM bis-Tris acetate, pH 7.5. The oxygenating gas contained 50% O₂/50% N₂. HbA attained complete oxygenation in less than 5 min, but the N102A(β) mutant required 15–20 min (that part of the curve not shown). The effect of chloride ion on the oxygen-binding affinity of Hb was determined in the presence of 0.5 M NaCl (Cl) as described in the text.

heme) of N102A(β) were used for the oxygen binding studies; at these concentrations, there is little dissociation into dimers in either the oxy or the deoxy conformations because the extent of dimerization was found to be very similar to that of HbA (in the micromolar concentration range). Even if there was some dissociation of the oxygenated recombinant Hb during measurement of the oxygen binding curve, it is unlikely that it occurred to a significant extent because a low oxygen affinity and signif-

Table 3. Oxygen binding parameters for HbA and N102A(β) Hb^a

	No NaCl	0.5 M NaCl	25% O ₂	50% O ₂
HbA				
P ₅₀ ^b (mm Hg)	5	11		
n ^b	2.4	2.4		
P ₅₀ ratio ^c (+Cl ⁻ /–Cl ⁻)			2.4	2.2
N102A(β)				
P ₅₀ ^b (mm Hg)	42	49		
n ^b	1.9	1.7		
P ₅₀ ratio ^c (+Cl ⁻ /–Cl ⁻)			1.1	1.2

^a P₅₀ values are expressed in mm Hg at the point of 50% saturation. For the N102A(β) mutant, the P₅₀ value was calculated from the oxygen equilibrium curve as shown in Figure 7 after approximately 90% O₂ saturation was attained in about 15–20 min. Cooperativity (n value) was calculated from the log-log plot of the saturation plot. The determinations were made on a modified Hem-O-Scan instrument at a Hb concentration of 2.4×10^{-3} M in heme and 37 °C.

^b Determined using 50% O₂/50% N₂ as the oxygenating gas.

^c Measurements were made separately with oxygenating gases containing either 25% O₂/75% N₂ or 50% O₂/50% N₂.

icant cooperativity were attained and these properties are not consistent with extensive dimerization (Turner et al., 1992). In contrast, the reported oxygen binding measurements for Hb Kansas were performed at dilute, i.e., micromolar Hb concentrations where there is extensive dissociation in the oxygenated state and loss in cooperativity. However, the natural mutant Hb St. Mande exhibits some cooperativity (Poyart et al., 1990). The results of the present study, as well as the previous report on the D99K(β) rHb (Yanase et al., 1994) indicate an advantage in using relatively high Hb concentrations for determination of the oxygen dissociation curves. Winslow et al. (1994) have also stressed the advantages of working with concentrated solutions of Hb to determine oxygen binding curves.

The oxygen binding properties of the N102A(β) mutant are in marked contrast to those found for the Asp-99(β) $\rightarrow$ Lys mutant (D99K(β)) (Yanase et al., 1994), which is at an adjacent site in the $\alpha_1\beta_2$ interface (see Jones et al. [1967] for a description of the properties of Hb Yakima, a natural human mutant at position 99(β)). Thus, in HbA, Asp-99(β) site is linked to Tyr-42(α) by a hydrogen bond in the deoxy conformation but not in the oxy conformation (Fig. 1). The results with the D99K(β) rHb were interpreted to indicate that substitution by a Lys disfavored the deoxy conformation by interfering with the normal linkage at this site in HbA. Thus, the large and unfavorable Lys replacement led to significant disruption of the $\alpha_1\beta_2$ interface and extensive dimerization in both the oxy and deoxy conformations, at least in the micromolar heme concentration range in which the studies were performed. In contrast, the choice of Ala for the substitution in the N102A(β) rHb was designed to preserve the integrity of this subunit interface; this objective was realized by the finding that the N102A mutant in the deoxy conformation was tetrameric under the conditions used for similar measurements for deoxy HbA.

Neither rHb D99K(β) nor rHb N102A(β) had a significant response to chloride with respect to a lowered oxygen affinity, but for completely different reasons. In the case of the D99K(β) mutant, the tendency to form dimers precluded its extensive functional interaction with chloride because the tetrameric structure, which is a prerequisite for such binding (Perutz et al., 1976, 1994; Bonaventura & Bonaventura, 1978; Manning et al., 1978; Perutz, 1990; Ueno & Manning, 1992; Ueno et al., 1993; Bonaventura et al., 1994; Manning, 1994), is disfavored in this recombinant mutant. Thus, there are three major functional chloride binding regions—the N-terminal/C-terminal region of the α -chains, the DPG pocket, and the central dyad axis. The latter region connects the first two regions at either end of the tetramer. The N102A(β) mutant is completely tetrameric in the millimolar concentration range used for the oxygen equilibrium studies, and the results suggest that the R $\rightarrow$ T equilibrium is shifted almost entirely to the T state even in the absence of chloride; hence, addition of chloride has little further effect.

Shih et al. (1985) reported a comprehensive study of the oxygen binding properties and effects of allosteric regulators of the natural mutant Hb with various substitutions at position 101 of the β -chain, between positions 99(β) and 102(β). They concluded that the size and the nature of the charge of the substitution were most important in determining the oxygen affinities of the mutant Hb. Our findings on the functional properties of the recombinant mutants D99K(β) and N102A(β), for which the amino acid replacements were chosen with specific objectives in mind, are in agreement with their conclusions.

Materials and methods

Reagents

All reagents were analytical grade. Restriction endonucleases and modifying enzymes (T4 ligase, etc.) were purchased from Boehringer Mannheim and Gene AmpR PCR reagent kits were from Perkin-Elmer/Cetus (Norwalk, Connecticut). The nucleotides used to make the mutation were purchased from Operon Technologies (Alameda, California).

Site-directed mutagenesis and PCR

The plasmid pGS189 and pGS389 that contained the full-length human α - and β -globin cDNAs under transcriptional control of the dual pGGAP promoter were used as gene sources (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). Adachi et al. (1993) employed a different strategy to obtain the sickle Hb mutation in this system. In our system, the 1.2-kb *Xho* I fragment containing a single GGAP promoter and the β -globin from pGS189 was inserted into the *Xho* I site on the *Escherichia coli* vector, pBluescriptR SK+, with opposite transcriptional direction of the *lac* promoter to create pBSK-BETA as a template for PCR mutagenesis. Two *Bam*H I sites are presented at 10 nucleotides upstream of the mutated site (Asn at position 102th) and located downstream of the *Xho* I site on the multiple cloning site encompassing the C-terminal region of the β -globin gene, respectively.

Taking advantage of these *Bam*H I sites, oligonucleotides containing a *Bam*H I site and including mismatches were used as primers in the PCR methodology to generate amino acid substitutions in the amplified DNA fragment, using pBSK-BETA as template. The following mixed oligonucleotides were prepared: 5'-CGTGGATCCTGAGXXXTTCAGGCTCTG-3'; the site indicated as XXX was substituted by A/T/T/G/G/C for Met, Ile, Arg, Ser, Phe, Leu, Trp, or Cys as the A mix primer, and by G/C/A/C/T/A for Asp, Glu, Ala, His, or Pro as the B mix primer were synthesized. The M13 reverse primer (5'-AACAGCTATGACCATG-3') was used as a primer for the other strand.

Approximately 1 μ g of pBSK-BETA DNA was used for input, and amplification was carried out with 20 cycles of 1 min denaturation at 94 °C, 2 min annealing at 37 °C, and 1.5 min extension at 72 °C. The concentrations of A mix primer or B mix primer and M13 reverse primer, dNTP, and Mg^{2+} in the reaction mixture were made according to the standard procedures suggested by Perkin-Elmer/Cetus.

The amplified DNA fragment (710 bp) was digested with *Bam*H I and purified from agarose gel by using a Pre A-gene kit (BioRad). The purified 580-bp *Bam*H I fragments were subcloned back to the *Bam*H I site of pBSK-BETA. The correct insertional direction of the mutated *Bam*H I fragment in pBSK-BETA(M) was determined by analysis with restriction enzymes *Sal* I or *Xho* I, and then by subjecting the digests to DNA sequence analysis. The mutation at the 102nd position of the β -globin gene was confirmed by this procedure as well as the absence of additional changes introduced by the PCR procedure.

After complete DNA sequence confirmation, the plasmid pBSK-BETA(M) was treated with *Xho* I to release the cassette with the mutated β -globin cDNA region, which was subsequently inserted into the expression vector pGS389 that had been digested with the same restriction enzyme. The correct orienta-

tion of the DNA fragment was again confirmed by restriction analysis and by DNA sequencing, and the recombinant plasmid pGS389-BETA(M) was transformed into *S. cerevisiae* GSY112 ciro strain. Transformants were selected on complete minimal agar plates without uracil.

Bacterial and yeast strains and growth conditions

The yeast expression system that contains the human α - and β -globin genes on the same plasmid was used. The yeast strains were grown in 10 culture flasks (2 L each) in the presence of ethanol as the carbon source. Expression of Hb was induced by the addition of 2% galactose for 24 h (Wagenbach et al., 1991; Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994).

Protein purification

Upon completion of growth, the yeast cells were saturated with CO gas, collected, broken by homogenization in a Bead-Beater in the presence of glass beads, and the initial purification step was achieved on carboxymethyl-cellulose (Whatman, CM-52). The second and final purification step was on HPLC with a Synchropak CM 300 (250 $\times$ 10 mm) column (Martin de Llano et al., 1993a, 1993b). The gradient was modified so that it consisted of 20–70% buffer B for 10 min, 70–88% buffer B for 30 min, and then 13 min of the 100% buffer B, each at a flow rate of 2.5 mL/min. The compositions of the buffers were A = 30 mM bis-Tris, 30 mM sodium acetate, 1 mM EDTA, pH 6.4; B = 30 mM bis-Tris, 150 mM sodium acetate, 1 mM EDTA, pH 6.6. All buffers were saturated with CO.

Analytical procedures

SDS-PAGE was performed on the recombinant N102A(β) mutant, as described previously (Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). Electrophoresis of the native protein was done on the Beckman Paragon system. Globin chains were separated by HPLC on a Vydac C-4 column (250 $\times$ 4.6 mm), also described previously, except that a different gradient was used, i.e., 47–50% B for 6 min, 50–60% B for 60 min, and then 60–70% B for 10 min, each at a flow rate of 1.0 mL/min (A = 0.1% trifluoroacetic acid [TFA]; B = 80% acetonitrile, 0.1% TFA). Isoelectric focusing was performed on the pH 6–8 Hb-Resolve system from Isolab.

Amino acid analysis of globin chains isolated by this procedure was performed on a Beckman 6300 instrument with System Gold data handling system. Tryptic peptide mapping was performed on the reduced, carboxymethylated globin chains, and separation of the tryptic peptides was carried out by HPLC, as described previously (Martin de Llano et al., 1993a, 1993b; Yanase et al., 1994). However, the gradient used for separation of the tryptic peptide of the mutant β -chain was changed to 0–15% B for 10 min, 15–55% B for 120 min, and then 55–100% B for 10 min, each at a flow rate of 1.0 mL/min on a Vydac D-18 column. Sequencing of this peptide was performed on an Applied Biosystem gas phase sequencer. Spectra of intact mutant and normal Hb were recorded on a Cary 2200 spectrophotometer. Hb concentrations are given on the basis of heme.

Mass spectrometric analysis

The purified N102A(β) mutant Hb tetramer was analyzed on a matrix-assisted laser desorption time-of-flight mass spectrometer constructed at Rockefeller University (Beavis & Chait, 1989, 1990). A small aliquot of the sample was applied to the top of probe, dried in air, and then inserted into the mass spectrometer for analysis. In this system, the Hb dissociates into its constituent α - and β -globin chains and heme is released. Horse heart myoglobin was the calibrant.

Determination of amount of Hb dimer

The binding to haptoglobin of dimer dissociated from the N102A mutant tetramer (132 kDa for the complex) and its separation from the unbound Hb tetramer (64 kDa) by use of a Centricon 100 filter was performed as described previously. In some experiments, quenching of fluorescence was used by procedures described previously (Yanase et al., 1994).

Determination of oxygen binding curves

For the recombinant and natural Hb (10 μ L, 2.4 mM in heme in 50 mM bis-Tris acetate, pH 7.5), the oxygen binding curves were measured at 37 $^{\circ}$ C on a modified Hem-O-Scan instrument (Manning, 1981; Yanase et al., 1994). The oxygen equilibrium of lower concentrations of Hb cannot be determined with this instrument. Prior to the analysis, the CO form of Hb, the ligand state in which the mutant Hb was purified to ensure that the heme was not oxidized, was converted to the oxy form by several exposures to incandescent light in an atmosphere of 100% O₂, as described previously (Manning, 1981). This conversion was considered complete when the A₅₄₀/A₅₆₀ ratio was 1.7. The oxygen equilibrium curves of the recombinant and normal Hb were measured in the absence or presence of 0.5 M chloride. The gas mixture used to achieve oxygenation was either 25% O₂/75% N₂, or 50% O₂/50% N₂ (Medgas).

Acknowledgments

We are grateful to Adelaide Acquaviva for the typescript. We thank Dr. Klaus Schneider and Dr. Brian Chait for molecular weight measurements and Dr. T.P. King for the use of his PCR apparatus. This work was supported by NIH grants HL-18819, HL-48018, and U.S. Army Contract DAMD17-94-V-4010.

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