Hemoglobin Porto Alegre forms a tetramer of tetramers superstructure

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Abstract

The effects of the mutation $\beta 9(\text{A6})\text{Ser} \rightarrow \text{Cys}$ on the interactions between the human hemoglobin molecules were investigated, and comparisons were made with other variants having an additional cysteine residue. In hemoglobin Porto Alegre (PA), the $\beta 9$ mutation induces polymerization by forming interchain disulfide bonds via the extra cysteine. The hemolysate from a heterozygote was separated by gel filtration into a tetrameric fraction and a higher-molecular-weight oligomeric fraction (30%). Reversed-phase high-performance liquid chromatography and electrospray ionization mass spectrometry (ESI-MS) under denaturing conditions showed that the tetrameric fraction contained only normal α - and β -chains, whereas the oligomeric fraction contained only normal α -chain and disulfide-linked β^{PA} dimer. Under native conditions, ESI-MS of the oligomeric fraction revealed a principal complex of mass 258,400 Da corresponding to a tetramer of tetramers, and 10% of minor components. Transmission electron microscopy corroborated this structure by showing four spheres of 140 Å diameter surrounding a central cavity. Equilibrium experiments on the oligomer at different concentrations, using gel filtration and dimer exchange experiments with metHbA-CN, showed that the tetramer of tetramers dissociates into smaller species, probably by breaking the dimer–dimer allosteric interface. None of the other variants investigated formed such a large oligomer.

Keywords: Hemoglobin; disulfide bridge; structure oligomer; oxygen transport

Hemoglobin Porto Alegre (Hb PA), $\beta 9(\text{A6})\text{Ser} \rightarrow \text{Cys}$, carries an extra thiol group oriented towards the exterior of the Hb molecule (Tondo et al. 1963). During storage of the

hemolysate, Hb PA spontaneously polymerizes by forming intermolecular disulfide bridges involving this cysteine residue (Tondo 1971). Other substitutions introducing a cysteine residue have been described in human Hb, but only Hb PA, Hb Mississippi $\beta 44(\text{CD3})\text{Ser} \rightarrow \text{Cys}$ (Adams et al. 1987), and Hb Ta-Li $\beta 83(\text{EF7})\text{Gly} \rightarrow \text{Cys}$ (Blackwell et al. 1971) are known to form polymers.

Tondo reported that in the fresh hemolysate only tetrameric Hb PA was found. In contrast, polymers were found in the aged hemolysate from homozygous and heterozygous patients (Tondo 1971, 1972). Using ultracentrifugation and osmotic pressure measurements, Tondo found that the polymer from a homozygote was composed of three tetramers of Hb PA, possibly with a closed ring structure

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Abbreviations: DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; Hb, hemoglobin; Hb A, human normal adult hemoglobin; Hb PA, hemoglobin Porto Alegre; Hb-CN, cyanmethemoglobin; Hb-CO, carbonmonoxyhemoglobin; DCL-Hb, diaspirin cross-linked hemoglobin; RP-HPLC, reversed-phase high-performance liquid chromatography; T, Hb tetramer; T_i , molecule of i tetramers; TEM, transmission electron microscopy; TFA, trifluoroacetic acid.

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(Tondo 1971). Surprisingly, when studying the aged hemolysate from a heterozygote, the same author described a polymer consisting of two Hb tetramers with one abnormal and one normal β -chain per tetramer (Tondo 1972). Likewise, animal Hbs have been reported that are able to polymerize in vitro such as mouse Hb (BALB/cj) (Bonaventura and Riggs 1967). In all cases, the cysteine residues were in an external position and the polymerization did not appear to alter the functional properties of the Hb.

Here we report on an investigation into the effects of the Cys substitution in Hb PA on the interactions within the oligomer which involve both covalent and noncovalent bonding. We also studied other Hb variants in which the substitution resulted in the introduction of an additional cysteine. Using gel permeation chromatography, electrospray ionization mass spectrometry (ESI-MS) and transmission electron microscopy (TEM), we determined the size and structure of the principal PA oligomer and propose a model for its spatial arrangement.

Results

Characterization of the Porto Alegre mutation

Hb PA was from a 30-year-old woman of Portuguese origin found to be heterozygous for this variant during routine antenatal screening. Table 1 shows the pattern of electrophoretic mobility observed under the various experimental conditions used for presumptive diagnosis in the Hb laboratory of the Henri Mondor hospital. The presence, in isoelectric focusing gel, of a small component with decreased pI, and, in urea-Triton PAGE, of an abnormal chain with increased hydrophobicity were the most striking features. The hematological parameters of the propositus were normal.

ESI-MS of the total globin revealed three components (results not shown). Two of these correspond to normal α - and β -chains of masses 15,126.4 Da and 15,867.2 Da, respectively. The third component of 31,764.5 Da suggests a

dimer composed of two β^{PA} -chains linked by a disulfide bridge (calculated mass 31,764.6 Da).

This substitution was confirmed by further structure analysis. Chromatography of the globin on the Partisil column led to the isolation of an abnormal peak significantly more hydrophobic than the normal β -chain. RP-HPLC separation of the peptides resulting from aminoethylation, and tryptic digestion of this fraction, showed that the normal $\beta\text{T-2}$ peptide was missing and replaced by a large abnormal peak eluting earlier. When this abnormal peptide was analyzed by tandem mass spectrometry (m/z 845.2), it was demonstrated to correspond to a $\beta\text{T-2}$ peptide (sequence $\beta\text{10-17}$) lacking the serine residue (β9) located at its N-terminus. This situation results from the formation of a new tryptic cleavage site following replacement of Ser β9 by Cys and aminoethylation.

Purification, size and structure of the oligomers

Gel filtration of the patient's hemolysate (2 mM on a heme basis) revealed the presence of two peaks (Fig. 1). One peak, amounting to about 70%, eluted at the expected volume for Hb tetramers (molecular weight 64,500 Da). Analysis of this fraction by RP-HPLC yielded only normal α - and β -chains (Fig. 2A). When analyzed under denaturing conditions by ESI-MS, this fraction showed only normal α - and β -chains. The other peak eluted from the gel filtration column over a volume range corresponding to molecular weights ranging from 120 to 350 kD. In the range of loaded volume, we estimated, from the top of the peak, a mean molecular weight of 288 ± 0.5 kD for this species. This value is consistent with a mixture of polymers assembled from four or five tetramers (4.5 ± 0.1). This high-molecular-

Table 1. Electrophoretic parameters of Hb Porto Alegre

Hb	Isoelectric focusing ^a	CAE ^b pH 8.6	Citrate agar	Urea 6M ^c		
				pH 9.0	pH 6.0	Triton X-100
Hb A	0.0	0.0	0.0	20.0	20.0	20.0
Hb PA oligomer	8.0	0.7	10.0	20.8	20.0	45.2

^a In mm relative to Hb A as in the isoelectric focusing gel reference map (Hocking 1997).

^b (CAE) cellulose acetate electrophoresis; migration values are measured according to a comparative scale (Schneider and Barwick 1986).

^c Values are given only for the β chain.

Fig. 1. Gel filtration profile of the hemolysate from a patient heterozygous for Hb PA. Experiments were performed at 25°C in 150 mM tris-acetate pH 7.5 as described by Manning et al. (1996). The flow rate was 0.4 mL/min, and the elution was monitored at 280 nm and 415 nm; 10–60 μL aliquots were applied at an Hb concentration of 2 mM on a heme basis, and eluted at a 0.4 mL/min flow rate. DCL-Hb (diaspirin cross-linked hemoglobin) was used as a control for undissociated tetrameric Hb. Hb Rothschild [$\beta\text{37(C3)Trp} \rightarrow \text{Arg}$] was used as a control for dimeric Hb.

Fig. 2. RP-HPLC profile of chain separation for (A) tetrameric Hb and (B) oligomeric Hb. The chromatograms were obtained before (*upper*) and after (*lower*) treatment with DTT, a thiol reducing agent. Each sample was applied to an Aquapore RP300 column, using an isopropanol-trifluoroacetic acid 0.2% gradient (Wajcman et al. 2001).

weight peak showed a shoulder towards lower molecular weights, indicating an equilibrium with smaller species.

RP-HPLC analysis of this fraction shows that only one peak eluted at the position of normal α -chain (Fig. 2B). When the globin sample was reduced by dithiothreitol (DTT), this fraction was split into two peaks corresponding to β - and α -chains, suggesting that the oligomeric fraction only contains disulfide-linked β^{PA} dimers (Fig. 2B). Figure 3A shows the MaxEnt deconvoluted spectrum, under denaturing conditions, of the oligomeric fraction from the top of the peak of the size exclusion chromatogram. A similar spectrum taken from the descending part of the peak shows that the oligomeric fraction contains only normal α -chain and β^{PA} dimer.

Figure 3B shows the ESI m/z spectrum of the oligomeric Hb under native conditions (pH 7.0), obtained at a declustering potential of 60 V. Dominant is a multiply charged series of ions (T_4) with 38–43 charges that correspond to a component with a mass of 258,670 Da. As the declustering potential was progressively increased to 100 V, the mass tended towards an asymptote at 258,400 Da. This latter mass is 0.18% higher than the calculated mass of $\alpha_8(\beta^{\text{PA-S-S-PA}}\beta)_{416}$ (257,934 Da) where α is the α -chain (15,126.4 Da), $\beta^{\text{PA-S-S-PA}}\beta$ is the dimer of PA chains (31,764.6 Da), and h is the heme group (616.5 Da). Multiply charged series corresponding to minor components (10% of

oligomeric Hb) comprising one to three tetramers (T_1 – T_3) and five tetramers (T_5) were also observed, where T is the tetramer $\alpha_2(\beta^{\text{PA-S-S-PA}}\beta)_4$ of calculated mass 64,483.4 Da.

The study of oligomeric PA by TEM shows that it appears as spheres of 140 Å diameter with a central cavity (Fig. 4). Some molecules have a crescent shape, suggesting a cleavage between two adjacent subunits (inset in Fig. 4).

Stability of PA oligomers

The concentration-dependent dissociation equilibrium of PA oligomers into tetramers was studied by gel filtration. Between 150 μM and 40 μM on a heme basis, the PA oligomers eluted at the retention time corresponding to a molecular complex formed by four tetramers. With a further decrease in concentration, the width of the peak at half height increased (Table 2), suggesting an equilibrium mixture of a more heterogeneous population of molecular species. At low concentration (4 μM on a heme basis), the maximum of the peak eluted at the retention time of a structure composed of two tetramers. Under these conditions, the peak was broader, indicating the presence of smaller species such as $\alpha_2\beta_2$ tetramers (Table 2). Note that at this concentration, Hb A dissociates into $\alpha\beta$ dimers and the peak width at half height is small, indicating the presence of only one species.

To test for exchange between PA oligomers and Hb A, we added a molar excess of HbA-CN to a sample of HbPA-CO. We observed a decrease in the fraction of the slow phase of CO recombination, as observed for a mixture of normal HbA-CO and HbA-CN. The results indicate, on the one hand, that bimolecular CO rebinding kinetics for oligomers were similar to those observed for Hb A; i.e., the tetramers function normally within the oligomer. On the other hand, this indicates an interaction of the oligomer with the HbA-CN, probably by a dimer replacement reaction (Fig. 5). Because the β^{PA} subunits are all tightly bound via S-S bonds, they are not free to separate from the overall oligomer. However, the oligomer may separate at the normal dimer interface and accept an external dimer. This could imply an increase in the overall molecular weight of the oligomer by the weight of one or two dimers. If the oligomer breaks in more than one place, then other smaller fragments might be produced. The gel filtration analysis of the HbA-CN and HbPA-CO mixture after flash photolysis showed the presence of intermediate species between two and four tetramers in size. The same experiment with a different ratio of HbPA-CO:HbA-CN was analyzed by gel filtration. Upon increasing the proportion of HbA-CN, the oligomer was eluted later and the width of the oligomer peak at half height became larger, indicating a higher heterogeneity of species (Table 3).

Fig. 3. ESI mass spectra of oligomeric Hb PA. (A) Under denaturing conditions, the sample (2.5 μ M) was analyzed in 1:1 acetonitrile:water containing 0.2% formic acid and introduced into the electrospray source at 5 μ L/min. Data were acquired for 3 min. (B) Under noncovalent conditions, the sample (5 μ M) was analyzed in water plus 10 mM ammonium acetate. The flow rate into the source was 4 μ L/min, and data were acquired for 10 min. In (B), T1–T5 correspond to multiply charged species comprising 1–5 tetramers [$\alpha_2(\beta^{\text{PA-PA}}\beta)_4$], respectively. The figures after the colons indicate the number of charges on the ions. “F” indicates tetramer ions that have lost an α -chain, probably in the ESI source, because their intensity increased with declustering potential.

Characterization of variant Hbs with an additional cysteine

The study by gel filtration of the hemolysate of the compound heterozygote patient for Hb Harrow [β 118(GH1) Phe $\rightarrow$ Cys] (Henthorn et al. 1999), and β^0 thalassemia showed that this variant Hb was eluted at a volume corresponding to the tetrameric Hb, except for a very small peak displaying an apparent molecular weight of 120 kD (Table 4). This result was checked by electrospray, which showed that the abnormal β -chain had the expected mass (15,823.2 Da). A small fraction of the abnormal β -chains (2%) was associated into dimers through an S-S bridge. The same observation was obtained for Hb Mississippi [β 44(CD3) Ser $\rightarrow$ Cys] (Adams et al. 1987); only a small fraction (3%) of this Hb forms oligomers. The sizes of these oligomers were higher and more heterogeneous than those observed for Hb Harrow, the oligomer peak being very wide. Contrary to

Fig. 4. Electron micrographs of oligomeric Hb PA negatively stained with 2% uranyl acetate. Scale bar 260 Å. Inset shows cleavage between two adjacent subunits.

Table 2. Effect of concentration on oligomeric fraction elution profiles

Heme concentration (μM)	Peak position (ml)	Peak width at half height (μl)	Molecular mass (kD)	Oligomer size ^a (xT) ^b
150	11.64	788	286.5	4.4
75	11.75	796	268.8	4.2
37.5	11.86	896	252.2	3.9
15	12.23	1332	203.6	3.2
10	12.54	1790	170.5	2.6
8	12.50	1832	173.9	2.7
4	13.27	780	111.4	1.7

^a The size of the oligomer was determined from a calibration curve using myoglobin (monomers), Hb Rothschild (dimeric form), and DCL-Hb (tetrameric form) as markers.

^b xT represents the number of tetramers and was obtained by dividing the apparent molecular mass by the molecular mass of tetramer (64.5 kD).

these two variant Hbs, Hb Ilmenau [$\beta 41(\text{C7})\text{Phe} \rightarrow \text{Cys}$] (<http://globin.cse.psu.edu/cgi-bin/hbvar>), Hb Arta [$\beta 45(\text{CD4})\text{Phe} \rightarrow \text{Cys}$] (Vassilopoulos et al. 1995), Hb Montfermeil [$\beta 130(\text{H8})\text{Tyr} \rightarrow \text{Cys}$] (<http://globin.cse.psu.edu/cgi-bin/hbvar>), and Hb Nunobiki [$\alpha 141(\text{HC3})\text{Arg} \rightarrow \text{Cys}$] (Shimasaki 1985)

A **B**

Fig. 5. Dimer exchange between HbPA-CO oligomers and HbA-CN. (A) Schema for the interaction between HbPA-CO oligomers (T_4) and Hb A-CN. Only one of the possible products is shown. (B) CO recombination kinetics after flash photolysis indicate an interaction resulting in less slow phase, characteristic of the deoxy Hb conformation. This is probably due to formation of [dimer-CN/dimer-CO] hybrids; because only the CO is photodissociable, less deoxy (or singly liganded) form can be produced.

Table 3. Effect of dimer exchange between oligomer PA-CO and HbA-CN on peak elution profiles

	Polymer			Tetramer	
	Peak position	Size ^a	Width of peak at half-height	Peak position	Width of peak at half-height
	ml	xT ^b	μl	ml	μl
HbPA-CO	11.27	5.5	844		
HbA-CN				14.07	872
HbPA:HbA ^c					
1:1	11.44	5.0	1020	14.18	804
1:2	11.65	4.4	1628	14.16	868
1:3	11.73	4.2	2020	14.14	848
1:4	11.98	3.6	1972	14.11	796
1:5	12.25	3.1	1748	14.14	864

^a See footnote of Table 2.

^b See footnote of Table 2.

^c The concentration of HbPA-CO oligomers and the HbA-CN was 70 μM on a heme basis; HbA-CN concentration was varied to obtain the different HbPA:HbA ratios.

did not form polymers; we observed only tetrameric forms by gel filtration.

Discussion

Previous studies have shown that Hb PA spontaneously oligomerizes without altering its functional properties (Tondo et al. 1974). The size of the oligomer in a homozygote has been described as being different from that in a heterozygote. In a homozygote, a compact closed ring comprising three tetramers linked by disulfide bridges was observed (Tondo 1971), whereas in a heterozygote, an octamer consisting of two asymmetric tetramers each with only one β^{PA} -chain was described (Tondo 1972). Tondo (1971) reported that Hb PA in red cells is present in the tetrameric form. He assumed that this could be due to the glutathione reductase activity that maintains the reducing environment, preventing the formation of disulfide bridges. He also found that the glutathione reductase activity was increased threefold in the erythrocytes of a homozygous Hb PA patient compared to normal subjects and that the red cells contain twice the amount of reduced glutathione (Tondo 1982). In another study, Martinez et al. (1977) reported normal glutathione reductase activity in heterozygous patients, and glutathione levels twice those of normal subjects. They proposed that glutathione maintains the Hb PA in the tetrameric functional form in the red cell. The oligomerization of the Hb PA was detected a few days after hemolysis of the red cells, the sample being stored at 5°C. Maximum oligomerization was observed after 50 days of storage.

We report here the study of a hemolysate from a heterozygote for Hb PA. The red cells were stored at -80°C for several months before analysis. Just after thawing and he-

Table 4. Characteristics of variant Hbs with an additional cysteine

Hb variants	Patient	Gel filtration of hemolysate		ESI-MS ^a denatured conditions dimers $\beta^S\text{-}\beta$	Localization in the tetramer ^b
		Tetramer %	Oligomer %		
Porto Alegre	heterozygous				
$\beta 9(\text{A6}) \text{ Ser} \rightarrow \text{Cys}$	A/PA	70	30	30%	external
Ilmenau	heterozygous				
$\beta 41(\text{C7}) \text{ Phe} \rightarrow \text{Cys}$	A/Ilmenau	100	0	nd	$\alpha 1\beta 2$ contact
Mississippi	heterozygous				
$\beta 44(\text{CD3}) \text{ Ser} \rightarrow \text{Cys}$	β^{thal} /Mississippi	97	3	nd	external heme contact
Arta	heterozygous				
$\beta 45(\text{CD4}) \text{ Phe} \rightarrow \text{Cys}$	β^{thal} /Arta	100	0	nd	external partly buried
Harrow	heterozygous				
$\beta 118(\text{GH1}) \text{ Phe} \rightarrow \text{Cys}$	β^{thal} /Harrow	98	2	2%	$\alpha 1\beta 1$ contact
Montfermeil	heterozygous				
$\beta 130(\text{H8}) \text{ Tyr} \rightarrow \text{Cys}$	A/Montfermeil	100	0	nd	internal
Nunobiki	heterozygous				
$\alpha 141(\text{HC3}) \text{ Arg} \rightarrow \text{Cys}$	A/Nunobiki	100	0	nd	$\alpha 1\alpha 2, \alpha 1\beta 2$ contacts

^a ESI-MS: The sample was the whole hemolysate.^b Sack et al. (1978).

(nd) Not determined.

molysis, the gel filtration profile showed the presence of tetrameric and oligomeric Hbs. Thus the kinetics of oligomer formation could not be compared to those observed by Tondo, because the preparation and storage conditions were different. In contrast to Tondo's results, both RP-HPLC and ESI-MS analysis show that all the PA β -chains are involved in the oligomer (30%). The tetrameric Hb fraction (70%) is free of the abnormal subunit. Moreover, the major form of the oligomer has a structure comprising four tetramers of composition $[\alpha_2(\beta^{\text{PA-s-s-PA}}\beta)h_4]_4$, according to the ESI-MS results under native conditions and TEM analysis. The species of mass 258,400 Da observed by ESI-MS appears to be the most stable form. Its mass is about 0.2% higher than the calculated mass of four tetramers (257,934 Da). This is consistent with measurements that have been made on several invertebrate Hb subunits (200–210 kD), where the mass excess ranged between 0.1 and 0.5%. Presumably, the higher than expected mass is due to additional water molecules and alkali metal ions (Green et al. 1999,2001). The ESI-MS results also indicate the presence of an oligomer incorporating a fifth tetramer (T_5 in Fig. 3B); this species is present at a relatively low level probably because of additional constraints.

Several results suggest that the PA oligomer structure is not frozen. First, minor groups of peaks of size T_1 – T_3 were observed by ESI-MS, indicating that different structures exist. Second, the filtration studies showed that, when the PA oligomer concentration was greatly decreased, the elution volume increased, indicating a decreased oligomer size. The width of the oligomer peak at half height also increased, indicating a higher heterogeneity of species (Table 2). Indeed, the minimum peak width occurred when one species was predominant (Dumoulin et al. 1997). The third piece of

evidence is that the oligomer interacts with HbA, probably through incorporation of $\alpha^A\beta^A$ dimers. These results can be compared to those of Tondo when mixing Hb A and Hb PA tetramers (Tondo and Reishl 1979), even though we did not find any oligomers with β^A subunits in the hemolysate from a heterozygote.

Using ESI-MS and TEM results, we propose the model presented in Figure 6. The oligomer is composed of four mutated tetramers. In this model, the formation of the fourth S-S bridge with the first tetramer would lead to a closed ring. Our TEM results for the PA oligomer support this hypothetical model. The dimensions of tetrameric hemoglobin are $50 \times 55 \times 65 \text{ \AA}$ corresponding to a mean diameter of $57 \text{ \AA}$. The calculated diameter of the oligomer central cavity ($64 \text{ \AA}$) is near to the experimental value ($40 \text{ \AA}$) measured on electron micrographs (Fig. 4).

Among the other variants studied with a cysteine substitution, Hb Ilmenau, Hb Arta, Hb Montfermeil, and Hb Nunobiki do not exhibit oligomers. In these variants, the substituted site is partly buried or internal (Hb Montfermeil) (Table 4). In contrast, Hb Harrow and Hb Mississippi exhibit low levels of oligomers (2–3%), lower than those observed for PA.

To our knowledge, Hb PA is the only variant of human Hb able to assemble into such high-molecular-weight oligomers through covalent bonds. This model is particularly interesting, because the oligomer PA exhibits functional properties similar to those observed for Hb A. In some primitive vertebrates and invertebrates such as annelids, the Hb exhibits deoxygenation-dependent self-association of monomers (Riggs 1998).

The Hb PA oligomer appears to be an interesting model for the realization of a blood substitute. Even at very low

Fig. 6. Three-dimensional model of the structure of HbPA oligomers. The Hb A crystallographic coordinates for the deoxy quaternary structure were taken from the file 4HHB (Protein Data Bank). The mutation $\beta 9(\text{A6})\text{Ser} \rightarrow \text{Cys}$ was introduced into the deoxy HbA using Hyperchem software. Four Hb PA molecules were imported using WebLab Viewer Pro (MSI) software, and positioned to form intermolecular disulfide bridges between the $\beta 9(\text{A6})\text{Cys}$ residues belonging to different tetramers. The α -subunits are shown in yellow, and the β -subunits are blue and green.

concentration, the size of PA remains higher than that observed for Hb A. Nevertheless additional constraints, such as crosslinking, would be needed to avoid separation at the dimer–dimer interfaces that do not involve the disulfide bonds.

Materials and methods

Hb variants

The structural abnormality was characterized as previously described (Wajcman et al. 2001). In brief, globin was prepared from whole hemolysate by the acid acetone method, and the polypeptide chains separated by RP-HPLC on a Partisil column (Whatman). After aminoethylation, the variant β -globin chain was digested with trypsin, and the resulting peptides were separated by RP-HPLC using a Vydac C8 column (The Separations Group). The substitution was characterized by tandem mass spectrometric analysis of the $\beta\text{T}2$ tryptic peptide.

Hb Ilmenau [$\beta 41(\text{C}7)\text{Phe} \rightarrow \text{Cys}$], Hb Arta [$\beta 45(\text{CD}4)\text{Phe} \rightarrow \text{Cys}$], Hb Harrow [$\beta 118(\text{GH}1)\text{Phe} \rightarrow \text{Cys}$], and Hb Montfermeil [$\beta 130(\text{H}8)\text{Tyr} \rightarrow \text{Cys}$] had been previously studied and were stored at -80°C . Hb Mississippi [$\beta 44(\text{CD}3)\text{Ser} \rightarrow \text{Cys}$] and Hb Nunobiki [$\alpha 141(\text{HC}3)\text{Arg} \rightarrow \text{Cys}$] were from the reference collection of samples at the Hemoglobin Laboratory of Henri Mondor Hospital (Crteil, France).

Purification of Hb oligomers

The oligomeric and tetrameric fractions were separated by gel filtration of the hemolysate on a Superose[®] 12 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated at 25°C with 150 mM tris-acetate pH 7.5 buffer as described (Manning et al. 1996). The chromatographic fractions were concentrated with 10 kD cutoff microconcentrators (Microcon YM-10, Millipore).

Studies of oligomeric and tetrameric Hbs

The subunit composition of the fractions obtained by gel filtration was determined by RP-HPLC using an Aquapore RP300 column (Brownlee) eluted with a gradient of isopropanol in 0.2% trifluoroacetic acid (TFA) (Wajcman et al. 2001).

Electrospray ionization mass spectrometry (ESI-MS)

Denatured conditions

Aliquots of the fractions isolated by gel filtration were diluted with 50% aqueous acetonitrile containing 0.2% formic acid. These solutions ($\sim 2.5 \mu\text{M}$ based on heme content) were introduced at $5 \mu\text{L}/\text{min}$ into the Z-Spray source of a Quattro Ultima mass spectrometer (Micromass) scanning over m/z 930–1180 (8 sec/scan). Data were accumulated for 3 min. Mass scale calibration employed the multiply charged normal α -chain peaks present in each spectrum. The m/z spectra were deconvoluted (m/z 970–1180) to present the data on a molecular mass scale using the Maximum Entropy (MaxEnt)-based software supplied with the instrument.

Native conditions

Initially, aliquots of the fractions isolated by gel filtration were diluted with HPLC-grade water. These solutions ($200 \mu\text{L}$, $\sim 5 \mu\text{M}$) were then desalted by manually shaking with $\sim 10 \text{ mg}$ of previously washed (twice with water) mixed bed ion exchange resin beads (AG 501-X8, BioRad) for 1 min. After pipetting the solutions from the beads, ammonium acetate was added to a 10 mM concentration and the pH was adjusted from ~ 6.7 to 7.0 by adding a trace of dilute ammonia solution. These solutions were introduced at $4 \mu\text{L}/\text{min}$ into the Z-Spray source of a time-of-flight instrument (LCT, Micromass). The source temperature was 110°C , and the desolvation gas was turned off. The pressure in the intermediate region between atmospheric pressure and high vacuum was increased to 7.0 mbar (normally $\sim 2 \text{ mbar}$) by throttling the backing line to the rotary pump. Data were accumulated over m/z 600–16000 for $\sim 10 \text{ min}$ at several declustering potentials (cone voltages) between 50 and 100 V. Masses of the noncovalently assembled species were determined from smoothed m/z spectra using peak top values by assuming the ions were protonated; i.e., $\text{mass} = n(m/z - H)$, where n is the number of charges on an ion and $H = 1.00794$. Mass scale calibration employed the $\text{Cs}_{(n+1)}\text{I}_n$ peaks from a separate introduction of CsI (2 mg/mL in 50% aqueous 2-propanol).

Transmission electron microscopy (TEM)

TEM was performed on purified Hb PA prepared as described above. Hb PA was diluted (1:900, final concentration $\sim 5 \mu\text{M}$ on a heme basis) in distilled water, and applied to a very thin carbon substrate supported on a microgrid stained with 2% (w/v) uranyl acetate solution as described by Valentine et al. (1968). The speci-

mens were observed with an electron energy of 80 keV, using a Jeol JEM-1200EX microscope (Jeol).

Kinetics of CO recombination

Kinetics of CO recombination were obtained after flash photolysis using 10-ns YAG laser pulses (Quantel) providing 160 mJ at 532 nm (Marden et al. 1988). Measurements were made at 25°C, 150 mM tris-acetate at pH 7.5, 100 μ M CO in 1 mm cuvettes, with observation at 436 nm.

Interaction of Hb PA oligomers with Hb A dimers was tested by mixing Hb PA-CO oligomers and HbA-CN. This method has previously been used to produce and study hybrid Hb tetramers: the two parent forms Hb-CO and HbA-CN can be mixed to produce the dimer-CO*dimer-CN hybrid (Marden et al. 1996). The dimer exchange occurs on the order of 1 sec for liganded Hb. These hybrid molecules show less of the slow (deoxy or T-state) CO rebinding, because only two of the ligands can be photodissociated.

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