Structural, Functional, and Subunit Assembly Properties of Hemoglobin Attleboro [α138 (H21) Ser → Pro], a Variant Possessing a Site Mutation at a Critical C-Terminal Residue†

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ABSTRACT: Hemoglobin Attleboro, a new α-chain variant with a substitution of proline for serine at position 138 (H21), was found to be a noncooperative high-affinity hemoglobin (P_{50} = 0.26 mmHg at pH 7 and 20 °C) which lacked an alkaline Bohr effect. Addition of 2,3-diphosphoglycerate (DPG) or inositol hexaphosphate (IHP) led to a decrease in oxygen affinity but to no alteration in either Bohr effect or cooperativity. Ligand binding kinetics studies revealed an overall rate of oxygen dissociation at pH 7.0 and 20 °C that was 2.7-fold slower than that for Hb A. At pH 8.5, the kinetic profile was identical with that at pH 7, confirming the absence of a Bohr effect for this variant hemoglobin. Measurement of the rate of oxygen dissociation with carbon monoxide replacement indicated a lack of cooperativity. Sedimentation velocity experiments yielded $s_{20,w}$ values of 2.8 and 4.3 for 65 μM solutions of oxyhemoglobins Attleboro and A, respectively (indicating an enhancement in the oxy dimer population of this variant). Studies of the carbon monoxide combination of this variant revealed an association rate 20-fold faster than that for Hb A; only in the presence of a 1000-fold molar excess of IHP was there a significant reduction in the overall rate. Rapid-scan and traditional stopped-flow experiments conducted in the Soret region demonstrated an alteration in the structure and rate of assembly of the deoxy tetramer of Hb Attleboro relative to that of Hb A. The abnormal properties of this hemoglobin variant can be attributed to major perturbations in the C-terminal region.

Of over 400 human hemoglobin variants reported to date (Bunn & Forget, 1986), only 126 have amino acid substitutions that reside in the α subunit. Since there are four α-globin chain genes and only one of these is normally subjected to genetic alteration, α-chain variants usually occur at a level not exceeding 25%. Hb[1] Attleboro [138 (H21) Ser → Pro], a new stable α-chain variant, is unusual in that it contributes only 11% of the total hemoglobin. Two other stable α-chain variants, Hb Fort Worth (Schneider et al., 1971) and Hb Spanish Town (Ahern et al., 1976), also contribute significantly less than 25% of the total hemoglobin. This has been attributed to possible defects in RNA processing (Schroeder, 1985).

Two other α-chain variants have been reported that contain substitutions in the same region of the protein as Hb Attleboro. These are Hb Bibba [136 (H19) Leu → Pro] (Kleihauer et al., 1968) and Hb Tokoname [139 (HC1) Lys → Thr]; the latter exhibits increased oxygen affinity (Harano et al., 1983). Indeed, abnormal oxygen affinity is generally related to the inability of the given variant hemoglobin to form a normal tetramer (Perutz, 1987). This appears to be the case for Hb Attleboro whose structural, functional, and tetramer assembly properties are presented here.

Experimental Procedures

Structural and Hematological Investigations of Hb Attleboro. Electrophoretic procedures for detecting abnormal hemoglobins and globin chains at acid and alkaline pH were performed as reported previously (Schneider, 1978). Hematologic data were collected by using a Coulter counter, and standard tests for unstable hemoglobins and the determination of HbF were employed (Singer et al., 1951; Rieder, 1970; Carrell & Kay, 1972). The abnormal hemoglobin was isolated on a Mono Q anion-exchange column (Pharmacia, Piscataway, NJ) (Moo-Penn & Jue, 1985). The abnormal α chain prepared by the Clegg procedure (Clegg et al., 1966) was modified with ethylenimine (Raftery & Cole, 1963) and digested with trypsin. The "core" peptides were prepared by acid cleavage of the chain at the Asp-Pro sequence, residues 94 and 95 (Landon, 1977). The two peptides generated, residues 1–94 and 95–141, were separated by column chromatography on Sephacryl S-200 gel (1.5 × 65 cm) in 20% acetic acid. The core fragment consisting of residues 95–141 was then digested with chymotrypsin (Smyth, 1967), and the peptides were resolved by reversed-phase HPLC. Peptide maps were prepared as previously reported (Bennett, 1967). After hydrolysis of the peptides in 6 N HCl for 20 h at 110 °C, amino acids were quantitated as phenylthiocarbamyl derivatives by reversed-phase HPLC on a Waters chromatograph.

Sedimentation Studies on Hb Attleboro. Centrifugation studies (Edelstein et al., 1970) were performed on the variant in the oxygenated form only. Samples were dialyzed overnight at 4 °C in 50 mM Tris buffer, then loaded into synthetic boundary, double-sector capillary type cells, and spun in a AnF aluminum rotor at 36 000 rpm in a Beckman Model E analytical ultracentrifuge thermostated at 20 °C. The moving boundary was scanned at 542 nm at 8-min intervals, and radial distances were determined. The sedimentation coefficient was

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[1] Abbreviations: DPG, 2,3-diphosphoglycerate; Hb, hemoglobin; IHP, inositol hexaphosphate.

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calculated and corrected to standard conditions.

Functional Investigations of Hb Attleboro. The oxygen dissociation curve of whole blood suspended in 150 mM phosphate buffer, pH 7.4, was monitored in a Hemox analyzer. For studies on purified Hb Attleboro and the control Hb A, organic phosphates were removed (Moo-Penn et al., 1988) and the oxygen affinity properties were determined by the manual tonometer method of Riggs and Wolbach (1956). The rates of ligand association and dissociation were determined by the classical method of Gibson (1959) as described by McDonald et al. (1985) in a stopped-flow rapid kinetic device at 20 °C using 20-mm path length cuvettes. Data were collected with a microcomputer-based OLIS 3820 system (On-Line Instrument Systems, Jefferson, GA).

Subunit Assembly of Deoxyhemoglobin Attleboro. Spectral changes accompanying the formation of deoxyhemoglobin tetramer were monitored by using rapid-scanning stopped-flow techniques. A Gibson Durrum stopped-flow apparatus utilizing a Harrick rapid-scanning spectrometer (Harrick Scientific Corp. Ossining, NY) was controlled by an OLIS 4100C operating system interfaced to a microcomputer. At every 0.5 nm, from 400 to 450 nm, the photomultiplier tube voltage was sampled eight times and averaged. These measurements, in conjunction with a prerecorded reference, were used to convert voltage to absorbance at each wavelength sampled. To determine the rate of deoxyhemoglobin tetramer formation, traditional stopped-flow techniques were employed as recently described (McDonald et al., 1985). A Kinetic Instruments stopped-flow apparatus furnished with a 20-mm cuvette, temperature-controlled at 20 °C, and integrated to a microcomputer based OLIS 4120AT system was employed for data reduction.

Results and Discussion

Structural and Hematological Studies of Hb Attleboro. In 1981, Hb Attleboro was detected in a 5 year old girl during a routine workup for mild anemia. The hematologic data on her family showed that, apart from mild anemia in the proposita and a slightly increased reticulocyte count (3%) in the girl and her father, all other hematologic measurements and physical examination results were normal. Hemoglobin electrophoresis showed the daughter and father to have an abnormal hemoglobin that migrated as a band between Hbs A and S on cellulose acetate at pH 8.3. An additional band between Hb A_2 and carbonic anhydrase, representing a variant Hb A_2, indicated that the substitution was in the α chain. On citrate agar electrophoresis the variant migrated between Hbs A and F. In denaturing mercaptoethanol–urea buffers at pH 6.0 and 8.9, only bands corresponding to the positions of α^A and β^A were observed, suggesting that a neutral amino acid substitution was involved. The solubility test and standard tests for unstable hemoglobins were all negative. The Hb A_2 concentration was 2.1% and that of Hb F was 1.2%.

Resolution of Hb Attleboro and Hb A was accomplished by chromatography on a Mono Q (Pharmacia) anionic resin (Figure 1). Quantitation of the hemolysate by this method gave 86.6% Hb A, 10.7% Hb Attleboro, and 2.7% Hb A_2. The abnormal α chain prepared from the purified Hb Attleboro fraction was modified with ethylenimine and subjected to acid cleavage (see Experimental Procedures). A resulting fragment containing residues 95–141 was digested with chymotrypsin, and the peptides were isolated by reversed-phase HPLC (Figure 2). An abnormal peptide, residues 137–141, was isolated. The amino acid composition was

Thr 1 (1), Ser 0 (1), Pro 0.7 (1), Lys 1 (1), Tyr 0.7 (1), Arg 1 (1)

137------ 138---------- 139------ 140-------- 141------

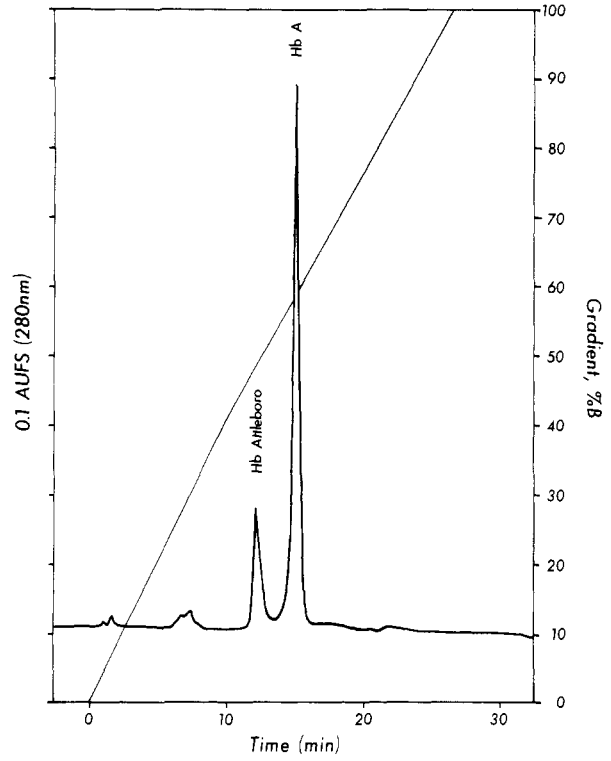

FIGURE 1: Chromatographic separation of Hb Attleboro on Mono Q anion-exchange resin. The column dimensions were 0.5 × 5.0 cm [HR 5/5 (Pharmacia)] and 1.6 × 10 cm [HR 16/10 (Pharmacia)] for analytical- and preparative-scale work, respectively. The flow rate was 1 mL/min. Buffer A was 50 mM Tris-HCl, pH 8.5, and buffer B was 50 mM Bis-Tris-HCl, pH 6.5. (For the analytical-scale separations only, 0.1% potassium cyanide was added to both buffers.) The two-step linear gradient was 0% buffer B at time zero to 40% buffer B at 10 min to 100% buffer B at 25 min.

These results are expressed as molar ratios and clearly show that at position 138 the serine has been replaced by proline.

Oxygen Equilibrium Properties of Hb Attleboro. The oxygen dissociation curve of whole blood containing only 11% Hb Attleboro was biphasic and shifted to the left compared to that of the curve for normal Hb A. This finding indicates that Hb Attleboro possesses an unusually high affinity for oxygen, since under most circumstances such a low percentage of abnormal hemoglobin would not markedly affect the oxygen dissociation curve of whole blood. Further studies of the oxygen equilibrium properties of purified Hb Attleboro substantiated these findings. Figure 3 and Table I reveal that stripped Hb Attleboro does possess a very high affinity for oxygen. In addition, the alkaline Bohr effect is absent, i.e., the P_{50} value, a measure of oxygen affinity, remains essentially unaltered with changes in pH. In addition, there is no cooperativity and the value of the Hill coefficient, n, is approximately 1.0 at all pH values (Table I). A nominal lowering of oxygen affinity was noted in the presence of a 100-fold molar excess of 2,3-diphosphoglycerate (DPG) or inositol hexaphosphate (IHP); however, the very small enhancement observed did not restore normal functionality to Hb Attleboro.

The equilibrium data for Hb Attleboro reveal a variant with very high oxygen affinity, no cooperativity, and no alkaline Bohr effect. In many respects, these functional properties resemble those of modified Hb A in which the last two or three C-terminal residues of the α chain have been removed enzymatically (Hb des-Arg 141, Tyr 140, and Hb des-Arg 141, Tyr 140, Lys 139) (Kilmartin & Hewitt, 1971). On the other hand, the functional characteristics of carboxypeptidase-treated hemoglobins are restored in the presence of organic phosphate

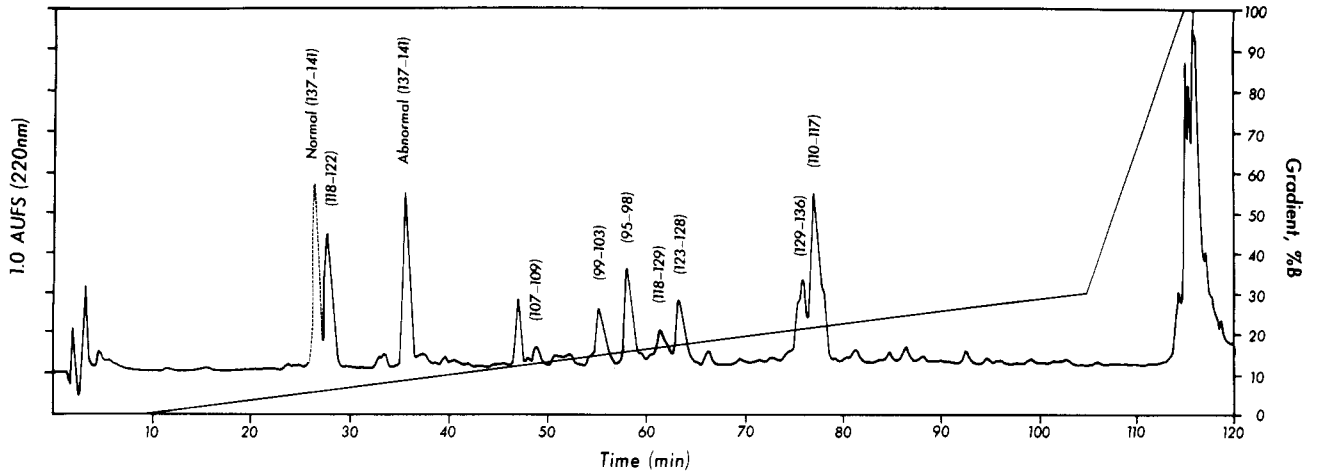

FIGURE 2: Separation of a chymotryptic digest of the core fragment, residues 95–141, by reversed-phase HPLC on a C18 column. The column was 0.39 × 30 cm and was operated at a flow rate of 1.2 mL/min. Buffer A was 0.1% TFA in water, and buffer B was 0.1% TFA in acetonitrile. The starting buffer was 0% buffer B from time zero to 10 min; a linear gradient was run to 30% buffer B at 105 min followed by a linear increase to 99.9% buffer B at 115 min.

Table I: Functional Properties of Hb Attleboro and Hb A[a]

	oxygen equilibrium		ligand binding kinetics		
	P_{50} (mmHg)	n	k (s^{-1})	k_4 (s^{-1})	l' (μM^{-1} s^{-1})
Hb Attleboro					
pH 7.0					
stripped	0.26	1.0	11	10	4.0
+DPG	0.50	1.2			
+IHP	0.78	1.0			0.87
pH 8.0					
stripped	0.21	1.0			
+DPG	0.30				
+IHP	0.75				
pH 8.5					
stripped			11		
Hb A[b]					
pH 7.0					
stripped	2.9	2.8	25	10	0.22
+DPG	12				
+IHP	40				0.10
pH 8.0					
stripped	0.71				
+DPG	2.5				
+IHP	10				
pH 8.5					
stripped			13		

[a] The values presented in this table were determined as described under Experimental Procedures. [b] Data for Hb A are derived either from experimental results or from Moo-Penn et al. (1984).

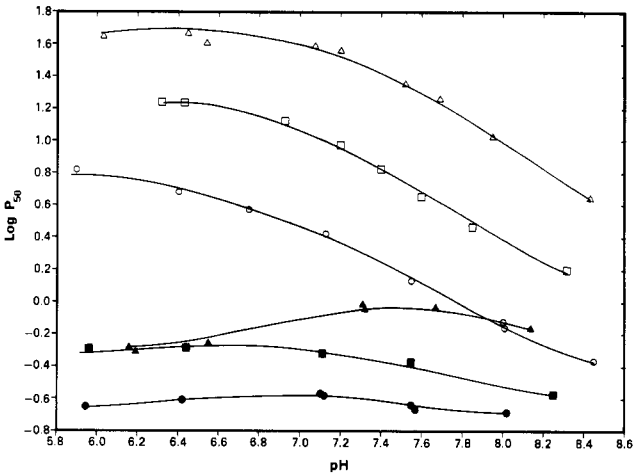

FIGURE 3: pH dependence of log P_{50} of Hb A and Hb Attleboro. The closed symbols represent Hb Attleboro stripped of phosphate ions (●) and in the presence of 100-fold molar excess (on tetramer basis) of DPG (■) and of IHP (▲). The open symbols represent the control Hb A results under comparable conditions. The concentration of hemoglobin was 60 μM in heme, in 50 mM Bis-Tris or Tris buffers (100 mM total Cl^-) at 20 °C. Note the unusual dependence of oxygen affinity on pH for Hb Attleboro in the presence of IHP; typically, as in the case of Hb A, the entire curve is shifted uniformly relative to the stripped molecule. However, for Hb Attleboro at acid pH values, the oxygen affinity is greater than expected and results in a distortion of the overall curve.

modifiers (Kilmartin et al., 1975), whereas Hb Attleboro shows no recovery of cooperativity or Bohr activity. This most certainly implies major disruptions in both the T and R forms of this molecule. Indeed, studies reported in this paper involving ligand binding, sedimentation, and subunit assembly support this premise (see below).

Ligand Binding Properties of Hb Attleboro. The combination of Hb Attleboro with carbon monoxide (l') was investigated at pH 7 and 20 °C (Table I) in the absence and presence of the potent organic phosphate modifier inositol hexaphosphate (IHP). The rate of binding of carbon monoxide to Hb Attleboro in the absence of IHP was found to be nearly 20-fold more rapid than that exhibited by Hb A under similar experimental conditions (Moo-Penn et al., 1984), a finding characteristic of a high-affinity hemoglobin. This ligand binding rate approached that determined for a dimer hemoglobin species (Andersen et al., 1971). The oxygen equilibrium studies of Hb Attleboro indicated a decreased response of this protein to organic phosphate modifiers; indeed, even a 1000-fold excess of IHP per tetramer of Hb Attleboro (250 μM IHP) caused only a 4.6-fold reduction in the carbon monoxide combination rate. This value is still nearly 10-fold faster than that reported for Hb A (Table I) and indicates a distorted deoxyhemoglobin Attleboro T-state (see below).

Kinetic measurements of ligand dissociation (Figure 4, Table I) also corroborated the oxygen equilibrium findings that Hb Attleboro is a high-affinity, noncooperative hemoglobin variant with no demonstrable Bohr effect. At pH 7 and 20 °C (see Figure 4A), the overall oxygen dissociation (k) time course was 2.7-fold slower for Hb Attleboro than for the Hb A control, indicative of a high-affinity hemoglobin. When the effect of pH on this rate constant was evaluated, the kinetic profile at pH 8.5 (Figure 4B) was found to exhibit a rate identical with that at pH 7. This finding indicates that the high-affinity Hb Attleboro possesses no Bohr effect and presumably is frozen in an R-state regardless of the absence or presence of protons. In addition, the rate of oxygen dissociation

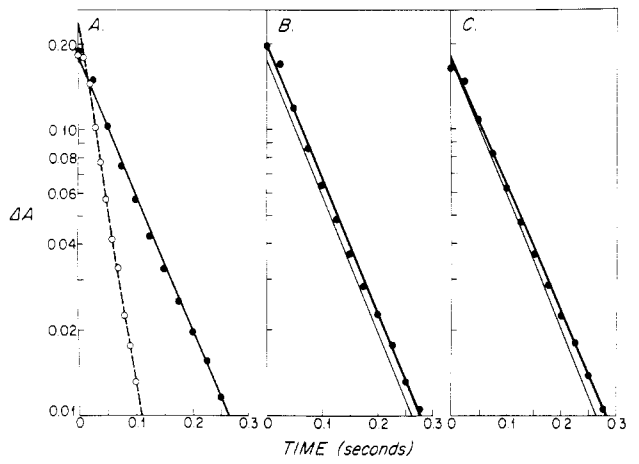

FIGURE 4: Time courses for oxygen dissociation. (A) Overall oxygen dissociation (k) of Hb Attleboro (●) and Hb A (○) in 50 mM Bis-Tris (100 mM total Cl^-), pH 7.0, 20 °C. (B) Overall oxygen dissociation (k) of Hb Attleboro in 50 mM Tris (100 mM total Cl^-), pH 8.5, 20 °C. (C) Oxygen dissociation with carbon monoxide replacement (k_4) of Hb Attleboro in 50 mM Bis-Tris (100 mM total Cl^-), pH 7.0, 20 °C. The kinetic time course of the overall oxygen dissociation at pH 7.0 for Hb Attleboro from panel A is transposed (lighter trace with no symbols) to panels B and C to aid comparison. The protein concentration was 45 μM in heme prior to mixing. A minimum of four independent determinations of five kinetic runs each was conducted at each condition. Rate constants were obtained by the method of least squares.

with carbon monoxide replacement (k_4) for Hb Attleboro at pH 7 and 20 °C was determined (Figure 4C). Within experimental error, the values of k and k_4 are identical, a feature diagnostic of a noncooperative R-state hemoglobin, possibly a dimer.

Dimeric Properties of Oxyhemoglobin Attleboro. The extreme high oxygen affinity and the accompanying lack of cooperativity of Hb Attleboro could be accounted for on the premise that this variant exists primarily in dimeric form even at protein concentrations known to favor a tetrameric species. In such a situation, the formation of mixed or asymmetric hybrids ($\alpha^A\alpha^{Attleboro}\beta^A_2$) would not occur (Park, 1973; Bunn & McDonough, 1974; McDonald et al., 1980, 1985). Indeed, the results of oxygen equilibrium experiments aimed at detecting such hybrid formation were negative (Figure 5). Mixtures containing 25:75, 50:50, and 75:25 Hb Attleboro:Hb A were analyzed, and for all mixtures, experimental and theoretically derived Hill plots were coincidental.

To further explore the premise that a large population of dimer species may account for the oxygen equilibrium properties exhibited by Hb Attleboro, sedimentation velocity experiments (Table II) were performed at a protein concentration (65 μM in heme) comparable to that used for functional studies. Under these conditions, oxyhemoglobin Attleboro had a $s_{20,w}$ value of 2.8–2.9; this value is identical with that determined by Edelstein et al. (1970) for hemoglobin dimers.

Since dimeric hemoglobin species are not readily crystallized, X-ray diffraction studies of the detailed structural features of these molecules are difficult. Such investigations have been successfully accomplished for several hemoglobin variants with either elongated or altered C termini by employing mixtures of variant and normal hemoglobins (McDonald et al., 1980; Moo-Penn et al., 1984, 1988). However, attempts at crystallizing Hb Attleboro (in the presence of absence of Hb A) were unsuccessful,[2] a finding

[2] Personal communication from Dr. Arthur Arnone, University of Iowa.

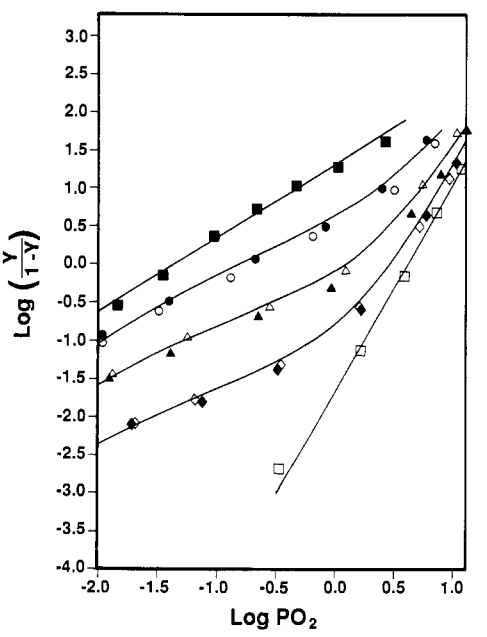

FIGURE 5: Hill plots for mixtures of Hb A and Hb Attleboro. Conditions were 50 mM Bis-Tris buffer (with no added Cl^-), pH 7.3 and 20 °C. Each mixture was run in duplicate: 25% Hb Attleboro (♦, ◊); 50% Hb Attleboro (▲, △); 75% Hb Attleboro (●, ○). Solid squares represent 100% Hb Attleboro; open squares represent 100% Hb A. The theoretical oxygen affinity of the mixtures in the absence of hybrid tetramers is shown by the solid traces.

Table II: Sedimentation Coefficients of Hb Attleboro and Hb A[a]

exptl conditions	$s_{20,w}$ Hb Attleboro	$s_{20,w}$ Hb A
65 μM oxyhemoglobin[b]		
pH 6.5	2.8	4.1
pH 7.4	2.8	4.2
pH 8.5	2.9	4.2

[a] The values presented in this table were determined as described under Experimental Procedures. Sedimentation studies were not attempted on the deoxygenated form of Hb Attleboro since it was uncertain that the strict anaerobic conditions required for this very high affinity variant could be maintained. Instead, subunit assembly experiments were performed during which the deoxygenated state of Hb Attleboro could be ensured (see text). [b] The concentration was calculated on a heme basis.

that appears consistent with both the oxygen equilibrium and centrifugation experiments presented here.

Subunit Assembly of Deoxyhemoglobin Attleboro. Deoxyhemoglobin formation can be monitored after rapid deoxygenation of dilute solutions of oxyhemoglobin (Kellett & Gutfreund, 1970; Gray, 1974; Ip et al., 1976; McDonald et al., 1985). A typical experiment is displayed in Figure 6A. A 5 μM (in heme) solution of oxyhemoglobin A in 50 mM Bis-Tris buffer, pH 7.0 (100 mM total Cl^-) and 20 °C, was mixed with a 0.5% solution of dithionite in a rapid-scan stopped-flow, apparatus. Soret spectral changes occurring as deoxyhemoglobin is assembled from dimers (present before deoxygenation) were monitored over time. The resultant scan profile readily illustrates that (at the completion of the deoxygenation process) a normal deoxyhemoglobin spectrum was observed. In contrast, rapid scanning of the Soret region (Figure 6A) subsequent to deoxygenation of Hb Attleboro (5 μM in heme) revealed a spectrum greatly altered from that of normal hemoglobin. Complete deoxygenation of Hb Attleboro resulted in a broadened spectrum exhibiting an absorbance value at 430 nm of only 80% that of normal deoxyhemoglobin A. This deoxyhemoglobin Attleboro spectrum was very similar to that reported for R-state hemoglobin

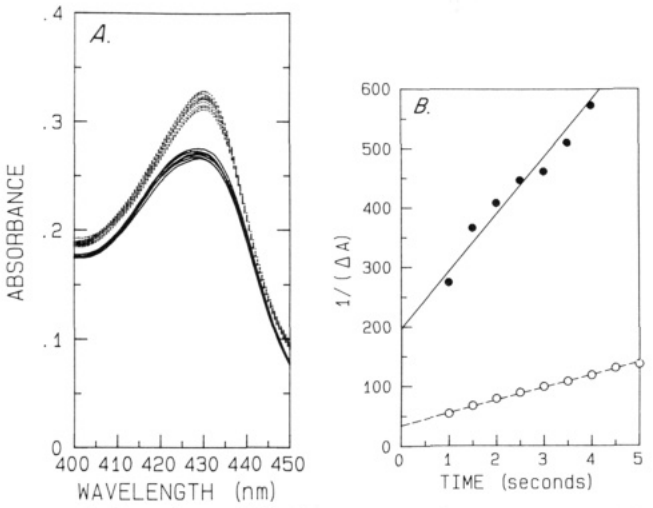

FIGURE 6: Deoxyhemoglobin dimer aggregation. An oxygenated, dilute solution of either Hb Attleboro or Hb A and an 0.5% solution of dithionite were rapidly mixed at 20 °C, and the slow second-order absorbance change following the rapid oxygen dissociation was monitored in a 20-mm cuvette (absorbance values are reported on a 1-cm basis). The experiments were conducted at a hemoglobin concentration of 5 μM on a heme basis, before mixing, in 50 mM Bis-Tris buffer (100 mM total Cl^-), pH 7.0. (A) Results of rapid-scan stopped-flow experiments. Scans taken between 1 and 5 s after mixing are presented in addition to a final 60-s scan which is equivalent to the deoxygenated spectrum as demonstrated by matched experiments in a Cary 2200 recording spectrophotometer. The upper series of traces depicts Hb A (···); the lower series represents Hb Attleboro (—). (B) Second-order plots derived from stopped-flow experiments. The dimer aggregation reaction was monitored at 430 nm. A minimum of three independent analyses consisting of three runs each were performed for Hb Attleboro (●) and Hb A (○). The rate of deoxy dimer association was calculated from the equation: $1/\Delta A_t = [({}^0k'_{\alpha\beta})(C_0t/\Delta A_0)] + 1/\Delta A_0$. The value of C_0, the concentration of $\alpha\beta$ dimers present before mixing (heme basis), was taken as 5 μM for Hb Attleboro (see text); for Hb A, C_0 was obtained by using $\Delta\epsilon = 12\ mM^{-1}\ cm^{-1}$.

subunits (Antonini & Brunori, 1971) and for noncooperative high-affinity hemoglobin dimers (Andersen et al., 1971). The spectral characteristics exhibited by Hb Attleboro in Figure 6A, in conjunction with the slow Soret absorbance change accompanying deoxyhemoglobin formation, strongly indicate that this α-chain variant is indeed an R-state *tetramer* species.

An alteration of the structure of deoxyhemoglobin Attleboro could influence the rate of the deoxy dimer aggregation reaction. Therefore, traditional stopped-flow experiments were conducted, and second-order plots of the slow absorbance changes are presented in Figure 6B. The rates of deoxy dimer combination were determined to be 0.10 and 0.25 $\mu M^{-1}\ s^{-1}$ for Hb Attleboro and Hb A, respectively. The 2.5-fold decrease in the rate of formation of deoxy Hb Attleboro tetramer is consistent with a disruption in the $\alpha_1\beta_2$ contact, an interface that is essential for correct tetramer assembly. Measurement of the dissociation of deoxyhemoglobin tetramers to dimers employing haptoglobin binding (Ip et al., 1976; McDonald et al., 1985) would have allowed the determination of an equilibrium dissociation constant for unliganded deoxy Hb Attleboro. These kinetic experiments were attempted with Hb Attleboro but were unsuccessful, apparently because the proline substitution disrupts the H-helix, a known binding site for haptoglobin (Kazim & Atassi, 1981). Similar studies of Hb des-Arg have revealed a decreased stability in the unliganded form, reflecting an altered C-terminal region (Turner et al., 1981).

The substitution of proline for serine at position 138 (H21) of the α chain appears to result in a general disordering of the C-terminal residues of Hb Attleboro, rendering them unable to participate in critical intersubunit contacts. The C-terminal residues of the α chain, Arg 141 (HC3) and Tyr 140 (HC2), are normally in contact with three residues, Tyr 35 (C1), Pro 36 (C2), and Trp 37 (C3) of the β chain, and form salt bridges that are critical to formation of a normal deoxy T-state structure (Perutz, 1987). Major destabilization of the T-conformation would result in disruption of normal hemoglobin function. Hence, the properties exhibited by Hb Attleboro are those of a high-affinity, noncooperative hemoglobin variant lacking a Bohr effect.

In addition to exhibiting abnormal oxygen-linked functional characteristics, Hb Attleboro also displays unusual tetramer assembly properties. The $\alpha_1\beta_2$ dimer interface is critical to the formation of both oxy and deoxy tetrameric structure, although the intersubunit contacts differ significantly (Fermi & Perutz, 1981). Numerous hemoglobin variants (Bunn & Forget, 1986) exhibit functional properties comparable to those of Hb Attleboro as a result of general structural disarray of the deoxy tetramer. What is unique about Hb Attleboro is that the proline substitution appears (at least at protein concentrations from 5 to 50 μM in heme) to have disrupted tetramer assembly of not only the deoxy- but also the oxyhemoglobin structure.

The presence of the hydrogen bond between Tyr 42 (C7) of the α chain and Asp 99 (G1) of the β chain is essential for the formation of the hemoglobin tetramer in the unliganded state (Perutz, 1987). Neither of these two residues form direct contacts with any of those of the β-chain C-helix region, the major area presumably affected by alteration of the α C-terminal region. Therefore, deoxy dimer aggregation to tetramer is not precluded and indeed was observed for Hb Attleboro. In contrast, the hydrogen bond between Asp 94 (G1) of the α chain and Asn 102 (G4) of the β chain stabilizes the oxyhemoglobin tetramer (Perutz, 1987). This bond could be significantly disrupted since the Asp 94 residue is also in contact with Trp 37 of the β chain whose orientation is critically determined by the repositioning of the α C-terminal region. Hence, assembly of an oxyhemoglobin tetramer from dimer may be impaired, and indeed, all studies presented here support a dimer structure for oxyhemoglobin Attleboro. A more detailed analysis of the molecular basis of Hb Attleboro subunit assembly is not feasible without corroborating X-ray diffraction studies. Future studies involving *molecular modeling* may permit an explanation of the perplexing properties of Hb Attleboro.

Our results show that in Hb Attleboro the substitution of a proline residue into the H-helix results in major conformational perturbations in the tertiary and quaternary structure. Apparently, the proline substitution in Hb Attleboro not only disrupts the H-helix per se but also results in a repositioning of the C-terminal residues, rendering them unable to participate in the formation of normal oxy- and deoxyhemoglobin tetramers.

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Prohormonal Cleavage Sites Are Associated with Ω Loops†

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ABSTRACT: Secretory peptides are generated from larger precursor proteins, or prohormones, by proteolytic cleavage at sites consisting of one or more basic amino acids. We have investigated the association of these cleavage sites with the various classes of secondary structure in the prohormones. In particular, we determined the association of cleavage sites with the newly defined category of Ω loops. We developed an algorithm for predicting the occurrence of such loops from the primary structure of the precursor and validated this procedure by comparison to crystallographic data. When this method was applied to prohormones, we found that about one-third of the cleavage sites previously assigned to reverse turns were actually associated with Ω loops. Moreover, sites that delimit secreted peptides are most often associated with loops and are concentrated in the neck regions of the loops. These data are interpreted in terms of a model in which the processing endoprotease interacts with two sites on the prohormone: a recognition site in the middle of a loop and the cleavage site at its neck.

Proteolytic processing plays an essential role in the generation of secretory peptides from their larger precursors. Cleavage is known to occur at lysine and arginine residues, most commonly at a Lys–Arg, Lys–Lys, or Arg–Arg pair but also at single residues or strings of three or four basic amino acids (Gluschankof & Cohen, 1987). Thus, the placement of cleavage sites in a protein is encoded in its primary structure. However, sites containing the same set of basic amino acids can be cleaved differentially, suggesting that some aspect of the structure of the region surrounding the cleavage site determines the kinetics of cleavage and, indeed in some cases,

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