Hemoglobin Raleigh (β1 Valine→Acetylalanine). Structural and Functional Characterization†

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ABSTRACT: Hb Raleigh is a unique human hemoglobin variant in which the N-terminal valine residue of the β chain is replaced by acetylalanine. The only other instance of a blocked β chain in adult human hemoglobin is found in the minor hemoglobin component, Hb A_{1c} (Bunn, H. F., Haney, D. N., Gabbay, K. H., and Gallop, P. M. (1975), *Biochem. Biophys. Res. Commun. 67,* 103). Hemoglobin A_{1c} is identical with Hb A except that the α-amino position of the β chain is bound to glucose through a Schiff base linkage. In addition, Schroeder et al. (Schroeder, W. A., Gua, J. T., Matsuda, G., and Fenninger, W. D. (1962), *Biochim. Biophys. Acta 63,* 532) have demonstrated that 10% of the γ chain of fetal human hemoglobin is acetylated at the α-amino position. Hb Raleigh constitutes approximately 45% of the total hemoglobin of hemolysates obtained from patients having the abnormality. In normal human hemoglobin, the α-amino groups of the two β1 valines are among the eight positively charged residues involved in the binding of organic phosphates and small anions (Arnone, A. (1972), *Nature (London) 237,* 146). Reduction of the number of cationic groups to six by the substitution of acetylalanine for valine results in a decreased oxygen affinity and a decreased interaction with organic phosphate cofactors. Surprisingly, the oxygen affinity of Hb Raleigh shows an increased sensitivity to NaCl. Neither the Bohr effect nor cooperativity of ligand binding is significantly altered by this substitution. The kinetics of ligand binding are somewhat more heterogeneous for Hb Raleigh than for Hb A, which suggests enhanced α-β chain differences. In addition, at comparable protein concentrations there is relatively less quickly reacting material in Hb Raleigh than in Hb A after complete flash photolysis of their CO derivatives. This is probably due to a decreased tendency for Hb Raleigh to dissociate into highly reactive subunits, a characteristic which may be associated with increased stabilization of the deoxy conformation.

Characterization of human hemoglobin variants continues to provide important information regarding the relationship between the structure and function of hemoglobin. Furthermore, investigation of mutations in critical areas of the molecule has substantiated many of the predictions made on the basis of x-ray crystallographic analysis. Such studies have enabled us to gain a more complete understanding of the molecular mechanisms of hemoglobin function.

Hb Raleigh (α_2 A β_2 1 Val→Ac-Ala) is a new variant which allows us to further study the organic phosphate binding site of the hemoglobin tetramer. The eight cationic groups normally involved in the binding of organic phosphates are the Val-1, His-2, Lys-82, and His-143 residues of the β chains (Arnone, 1972). Naturally occurring variants involving all of these positions except the β1 Val position have been described (Bare et al., 1974; Bonaventura et al., 1975a,b, 1976; Jensen et al., 1975; Lorkin et al., 1975; Moo-Penn et al., 1976). In Hb Raleigh, the substitution of alanine for valine does not lead to a charge change. However, the α-amino group is acetylated in Hb Raleigh and therefore cannot contribute to the charge cluster of the cofactor binding site as it does in Hb A.

In this paper we report the structural characterization of Hb Raleigh and present data on the functional properties of this abnormal hemoglobin.

Experimental Procedures

Electrophoretic Studies. Electrophoretic methods used in the detection of abnormal hemoglobins have previously been described (Schmidt and Brosious, 1976). Electrophoresis was performed on cellulose acetate strips with a Tris–EDTA–boric acid[1] buffer (pH 8.4) (Supre-heme buffer,[2] Helena Laboratories), and on citrate agar plates with a 5 mM sodium citrate buffer (pH 6.2).

Globin chains were also analyzed on cellulose acetate membranes and strips (Super Sephraphore and Sepraphore X, Gelman Instrument Co.) under alkaline and acid conditions (Schneider, 1974, with modifications). In the alkaline globin procedure (pH 8.6), 50 μL of hemolysate was combined with 50 μL of 2-mercaptoethanol and 50 μL of a 6 M urea solution made with Supreheme buffer. The mixture was incubated at 22 °C for 30 min and applied to a membrane (10 × 5.5 cm) which had been soaked for 30 min in 300 mL of the same buffer containing 2.4 mL of 2-mercaptoethanol. The membrane was electrophoresed at 150 V at 1–2 mA for 120 min. In the acid

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[1] Abbreviations used are: Hb, hemoglobin; Ins-P_6, inositol hexaphosphate; DPG, 2,3-diphosphoglycerate; Tos-PheCH_2Cl, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; Bistris, 2,2′-bis(hydroxymethyl)-2,2′,2″-nitriloethanol; Ac, acetyl; NMR, nuclear magnetic resonance; Th, thermolytic; Cl_3AcOH, trichloroacetic acid; Me_4Si, tetramethylsilane; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

[2] Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

globin procedure, the same buffer was used but the pH was lowered to 6.0 with a 30% solution of citric acid. A 40-μL sample of hemolysate was incubated with 20 μL each of buffer and 2-mercaptoethanol at 20 °C for 30 min; it was applied to a strip (7.5 × 5.5 cm) and electrophoresed under the same conditions as above. A solution of 0.5% Ponceau S in 5% acetic acid and 5% Cl_3AcOH was used for staining the membranes and strips.

Isoelectric focusing was performed on 4% polyacrylamide gels containing 2% Ampholine (LKB 6-8) as described by Drysdale et al. (1971). The gel slab (10 × 7.5 × 0.3 cm), made with a commercial apparatus (Ortec), was electrophoresed at 4 °C for 1 h at 150 V and 25 mA. After this period the voltage was increased to 200 V for 1 h and finally to 250 V for 3 h. The protein bands were stained with a stain fixative derived from Coomassie Blue, which gave good definition of bands and did not require gel destaining (Malik and Berrie, 1972).

Structural Studies. Hemoglobins were separated by ion-exchange chromatography (Huisman et al., 1958; Huisman and Dozy, 1965). Heme was removed by the acid-acetone procedure, and globin chains were isolated according to the method of Clegg et al. (1966). The abnormal chain was reduced with dithioerythritol, aminoethylated (Raftery and Cole, 1963), and digested with $TosPheCH_2Cl$-trypsin (Smyth, 1967). The peptides were resolved by chromatography on a column (0.9 × 23 cm) of Aminex A-5 (Bio-Rad Laboratories) with a pyridine-acetate gradient (Jones, 1970). Rechromatography was carried out on Dowex 50-X4 or Dowex 1-X2 (Schroeder, 1972). Peptide maps were prepared by chromatographing in pyridine-1-butanol-acetic acid-water (10:15:3:12) or isoamyl alcohol-pyridine-water (7:7:6) and then electrophoresing at pH 4.7 or 6.4 at a potential of 35 V/cm for 2.5 h (Bennett, 1967). The maps were air-dried and sprayed with a solution of 6 mg of fluorescamine in 100 mL of acetone; the peptide spots were then visualized under ultraviolet light.

Peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h in vacuo and analyzed on a Beckman Model 121 amino acid analyzer by the method of Spackman et al. (1958). Sequence analysis was carried out on a Beckman 890C sequencer according to the programs provided by the manufacturer (Beckman Instruments, 1975).

βTp I was digested with thermolysin in 0.05 M Tris buffer (pH 8.0) containing 2.5 mM $CaCl_2$ to produce peptides for mass spectrometry and NMR studies. The enzyme to peptide ratio was 1:6 by weight. The mixture was incubated for 6 h at 37 °C, and the reaction was terminated by adding glacial acetic acid. The thermolytic peptides were separated on Aminex A-5 resin (Jones, 1970) and then desalted on a column of G25 fine Sephadex in water.

Mass Spectrometry and NMR Studies. The mass spectra were obtained on a Varian MAT CH-5 DF instrument equipped with a combined field desorption, field ionization, electron ionization source. The emitters were 10 μm tungsten wires activated at high temperature in a Varian apparatus, essentially by the procedure of Schulten and Beckey (1972). The peptide was dissolved in 100 μL of a methanol-water solvent (20:80) and applied to the emitter by the dipping technique (Beckey, 1969). The anode heating current was 21 mA. The threshold value was 10%. Mass assignments were made by comparison with the electron impact spectrum of perfluorokerosene.

The proton NMR spectra were obtained at 100 MHz on a JEOL PFT-100 spectrometer with an internal deuterium lock. The peptide sample was dissolved in ^2H_2O. The spectrum resulted from Fourier transformation of 5000 co-added free induction decays which resulted from $\pi/2$ pulses given with a recycle delay of 0.5 s. The rapid repetition rate (and resultant loss of resolution) was used to help suppress the residual HDO resonance because of severe dynamic range problems associated with the diluteness of the peptide sample. The final 2048 point data table was multiplied by a negative exponential to improve the signal-to-noise ratio which resulted in a further 3-Hz line broadening.

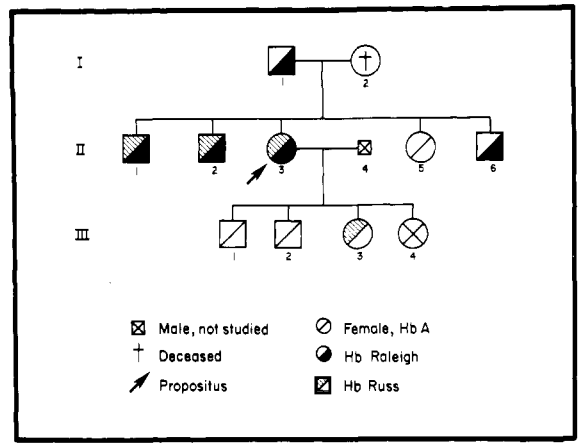

FIGURE 1: The pedigree of the family with Hb Russ and Hb Raleigh.

Oxygen Equilibrium Studies. The oxygen equilibrium studies were done by the spectrophotometric method of Riggs and Wolbach (1956). The purified hemoglobins were stripped of organic phosphates by passage through a column of mixed bed resin (AG 501-X8, Bio-Rad Laboratories). The hemoglobin in the tonometer was deoxygenated by alternately evacuating and flushing with nitrogen. Approximately 60 μM hemoglobin solutions in 0.05 M Bistris or Tris buffers were used. The tonometers were incubated for a minimum of 12 min at 20 °C. The preparation of DPG and Ins-P_6 is described elsewhere (Bonaventura et al., 1974b). These allosteric effectors were added in a 100-fold molar excess over tetramer. Hb A purified from the same column was used for all control functional studies.

Kinetic Experiments. Rapid mixing experiments were performed with a Gibson-Durrum stopped-flow apparatus equipped with a pneumatic drive. The 486.10- and 656.28-nm lines from the hydrogen lamp were used to check the wavelength calibration. The apparatus has a deadtime of about 2.3 ms. Flash photolysis experiments were performed as previously described (Bonaventura et al., 1974a). Approximately 0.05% sodium dithionite (Merck) was used to deoxygenate hemoglobin in kinetic experiments. Data were analyzed with a PDP-11/E computer (Digital Equipment Corporation) and a data acquisition and storage device (DASAR, American Instruments Co.).

Enzymes were purchased from Worthington Biochemical Co., and DPG and Ins-P_6 were obtained from Sigma. All other chemicals used in this study were of analytical grade.

Results

Family Studies. Figure 1 shows the pedigree of the family members who were studied. The original propositus was a 41-year-old female Caucasian who was heterozygous for Hb Russ (α51 Gly→Arg), a variant previously characterized by Reynolds and Huisman (1966), and for Hb Raleigh (β1 Val→Ac-Ala). Two male siblings showed the same inheritance pattern. The sister of the propositus had Hb A, and her other brother had only the Hb Raleigh gene. The family study revealed that the propositus' father contributed the Hb Raleigh

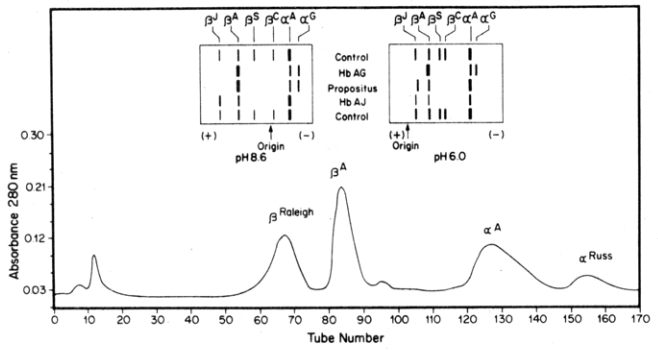

FIGURE 2: Globin chain analysis (upper panel) of the propositus' hemolysate. Experimental details are included in the text. The abnormal α chain (Hb Russ) and β chain (Hb Raleigh) separate under alkaline and acid conditions, respectively. The control was a synthetic mixture of Hbs A, S, C, and J. Diagrammatic representations were made from cellulose acetate membranes and strips. The lower panel shows qualitative separation of the globin chains from a hemolysate. The globin was applied to a CM-cellulose column (2.0 × 15 cm), and the chains were separated by using a linear gradient of 500 mL each of 5 mM and 0.04 M potassium phosphate buffers (pH 6.7). Both buffers were made with a solution of 8 M deionized urea, 0.05 M in 2-mercaptoethanol. The absorbance was monitored continuously at 280 nm.

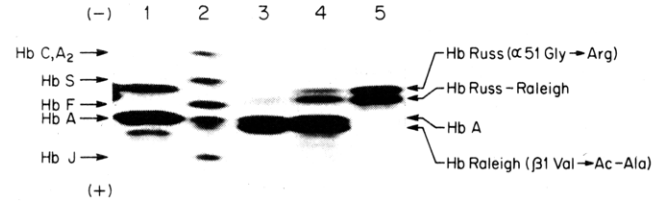

FIGURE 3: Isoelectric focusing patterns of (1) Hb Russ, (2) Hb control, (3 and 5) partially purified hemoglobin fractions from the propositus' hemolysate, and (4) the propositus' hemolysate. This experiment was done under oxygenation conditions. Both KCN and CO were added to the samples to convert methemoglobin to cyanomet- and carboxyhemoglobins which electrofocus with oxyhemoglobins.

gene and her mother, who was not examined, probably contributed the Hb Russ gene. The hematologic values obtained on the propositus were within normal limits, and no clinical symptomology related to the presence of these abnormal hemoglobins was observed.

Electrophoretic Studies. Electrophoresis of a hemolysate from the propositus on cellulose acetate (pH 8.4) revealed two bands, one with a mobility like Hb A and the other like Hb S. When the same sample was subjected to citrate agar electrophoresis (pH 6.2), two bands were also observed, one with a mobility like Hb A and the other like Hb F. Visual examination of the bands indicated values of approximately 20 and 40% for the abnormal hemoglobins present at the Hb S and Hb F positions, respectively. The results of our investigation of these hemoglobins show that the abnormal hemoglobin migrating in the Hb S position is Hb Russ (α51 Gly→Arg) and the abnormal hemoglobin migrating in the Hb F position is Hb Raleigh (β1 Val→Ac-Ala). The value of Hb F was 0.3% as determined by the alkali denaturation test.

Globin chain electrophoresis at pH 6.0 revealed αA and βA chains, as well as a β chain migrating like the β chain of Hb J (β16 Gly→Asp). However, at pH 8.6, the mutant β chain was not observed; instead, an α chain migrating like the α chain of Hb G Philadelphia (α68 Asn→Lys) was seen, in addition to the αA and βA chains (Figure 2, upper panel). The presence of more than one abnormal globin chain was substantiated by the Clegg et al. (1966) globin preparative procedure (Figure 2, lower panel). These chains were used for structural characterization to determine the sites of amino acid substitution.

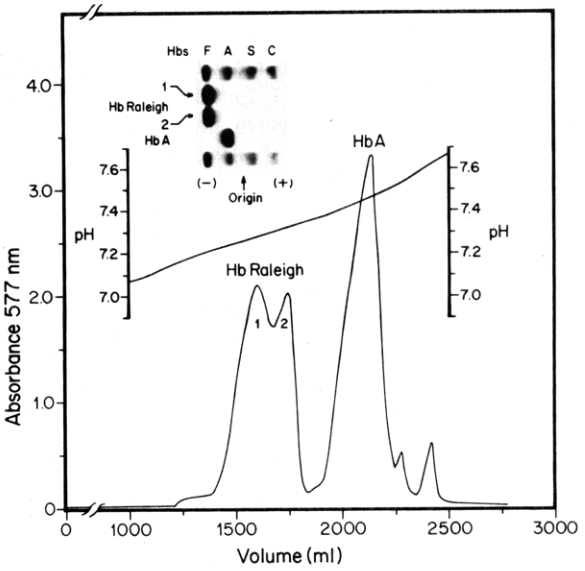

FIGURE 4: Chromatographic separation of Hb Raleigh on CM-cellulose. A hemolysate (1.5 g) was applied to a column (1.6 × 68 cm) operated at a flow rate of 21 mL/h. The separation was done at 4 °C. The insert panel shows the citrate agar electrophoretic pattern of the various pooled fractions. Hb Raleigh migrates in the position of Hb F on citrate agar.

On the basis of the above observations, isoelectric focusing experiments were done to determine whether hybrid tetramers occurred in vivo. These results are shown in Figure 3. Position 1 represents a sample from an individual who is heterozygous for Hb Russ; position 2 is a synthetic mixture of hemoglobins; and positions 3 and 5 are hemoglobin fractions from the propositus (position 4) which were purified partially by chromatography on DEAE-Sephadex. The distribution of hemoglobin bands indicates that Hb Russ–Raleigh with a structure of α_2 Russ–β_2 Raleigh is the only type of hybrid that is detected. Hybrids composed of four types of chains were not detected.

Chromatographic Separation. Hb Raleigh used for functional studies was purified from a blood sample obtained from the propositus' brother who is heterozygous only for this abnormal hemoglobin (Figure 1, II-6). The elution profile shows that the abnormal hemoglobin can be clearly isolated from Hb A. Although it elutes as a split peak, citrate agar and globin chain electrophoretic analyses indicate that both fractions contain only Hb Raleigh (Figure 4). These results confirm the absence of nonacetylated Hb Raleigh, which would be expected to behave like Hb A. The percentages of hemoglobins from this separation were 45.2% Hb Raleigh, 52.7% Hb A, and 2.1% Hb A_2. The percentage value for Hb Raleigh might be slightly high because some hemoglobin degradation products migrate in this region.

Structural Analysis. The variant α and β chains isolated from the blood of the propositus were used for structural analysis (Figure 2). The Hb Russ mutation (α51 Gly→Arg) was confirmed by isolation of tryptic peptides αTp VIa (residues 41–51) and αTp VIb (residues 52–56) on Aminex A-5 resin (Table I). Tryptic peptide αTp VIb cochromatographs with αTp IV, whereas αTp VIa chromatographs between a peak containing αTp III and αTp XIIa and a peak containing αTp X. The amino acid composition of the abnormal peptides eluted from the peptide map also gave similar results.

The tryptic digest of the abnormal β chain of Hb Raleigh was examined by peptide fingerprinting. The distribution of

TABLE I: Amino Acid Composition of αTp VIa and VIb from Hb Russ (α51 Gly→Arg).

	αTp VIa		αTp VIb + IV	
Amino acid	Calcd	Found	Calcd	Found
Lys			1	0.9
His	2	1.9	1	1.0
Arg		0.9	1	1.0
Asp	1	0.9		
Thr	1	0.8		
Ser	1	0.9	1	0.8
Glu[a]			4	3.9
Pro	1	0.9		
Gly	1	0	3	2.8
Ala			5	4.8
Val			2	2.3
Leu	1	1.0	1	1.0
Tyr	1	0.8	1	1.0
Phe	2	1.9		

[a] Amides are converted to the acid during hydrolysis. Peptides were hydrolyzed in 6 N HCl at 110 °C in vacuo for 24 h. The results are expressed as molar ratios.

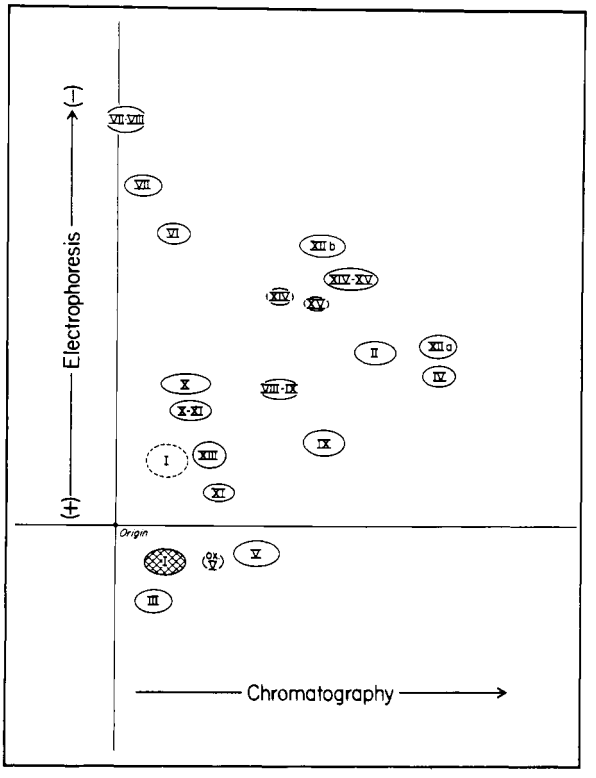

FIGURE 5: The peptide map of a tryptic digest of Hb Raleigh. Chromatography was performed in isoamyl alcohol–pyridine–water (7:7:6), and electrophoresis was carried out at pH 6.4.

peptides was normal except that βTp I was displaced anodally (Figure 5). The peptides were also separated by column chromatography, and βTp I coeluted with βTp III and XIII instead of in its normal position between βTp II and XIV–XV (Figure 6, lower panel). βTp I was purified by rechromatography on Dowex 1-X2 (Figure 6, upper panel) or on Dowex 50-X4. The amino acid composition is shown in Table II. The data reveal that valine is replaced by alanine. Identical results were obtained after elution and amino acid analysis of the abnormal peptide from peptide maps. Since the similar amino acid substitution cannot account for the observed electrophoretic and chromatographic behavior of the abnormal hemo-

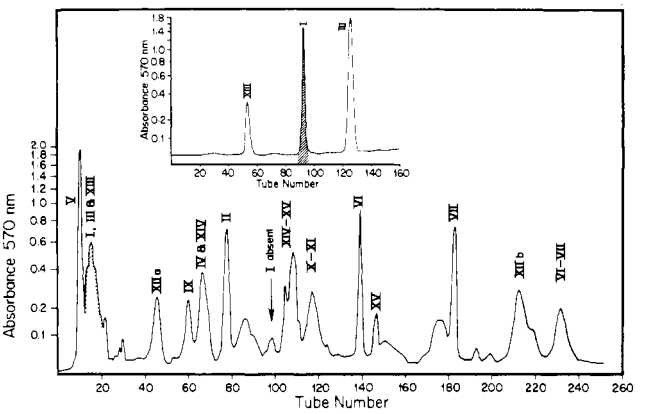

FIGURE 6: The separation of a tryptic digest of Hb Raleigh by column chromatography on Aminex A-5. Abnormal βTp I coeluted with β peptides III and XIII. The insert panel shows the purification of βTp I by rechromatography on Dowex 1-X2. The column (0.9 × 60 cm) was operated at 37 °C at a flow of 30 mL/h. Approximately 6% of the sample was removed, hydrolyzed with 5 N NaOH, neutralized, and then reacted with ninhydrin for continuous monitoring at 570 nm.

TABLE II: Amino Acid Composition of βTp I and Its Constituent Thermolytic Peptides from Hb Raleigh (β1 Val→Ac-Ala).[a]

Amino	βTp I		Th 1		Th 2	
acid	Calcd	Found	Calcd	Found	Calcd	Found
Lys	1	1.0			1	0.9
His	1	0.8	1	1.0		
Thr	1	0.9			1	1.0
Glu	2	2.1			2	2.0
Pro	1	0.9			1	1.0
Ala		0.9	1	0.9		
Val	1	0				
Leu	1	1.0			1	0.9

[a] βTp I was obtained by rechromatography on Dowex 1-X2, and the thermolytic peptides were separated on Aminex A-5. Hydrolysis was performed in 6 N HCl in vacuo at 110 °C for 24 h. The results are expressed as molar ratios. Other experimental details are included in the text.

globin and peptide, additional studies were undertaken to determine whether an N-terminal blocking group was present. Furthermore, indirect evidence to support the notion of the existence of such a group was inferred from the unsuccessful attempts at automated sequence analysis of the intact chain.

Identification of the N-Terminal Blocking Group. Field desorption mass spectrometry and nuclear magnetic resonance were used to determine the chemical nature of the blocking group. The first technique has been applied to sequencing small peptides when there is sufficient fragmentation of the peptide backbone (Asante-Poku et al., 1975). In Hb Raleigh, the dipetide X-Ala-His was prepared by thermolytic cleavage of βTp I (Table II). Assignments of masses in the spectrum are consistent with the structure of Ac-Ala-His (mol wt 268). In the field desorption mass spectrum, there is a mass peak at *m/e* 268 and at 269 (M + H). Mass peaks 253 (M + 2H − OH or M − CH_3), 225 (M + 2H − COOH or M + H − COO), 112 ((CH_3COAla) − 2H), 60 (CH_3CONH + 2H), 58, and 43 are also related to the sequence of the dipeptide (Figure 7).

^{1}H NMR was also used to confirm the blocking group because of the distinct, sharp three-proton methyl resonance unsplit by scalar couplings that is associated with the acetyl group. The ^{1}H NMR spectrum of the blocked dipeptide shows a sharp intense resonance approximately 2.0 ppm from Me_4Si

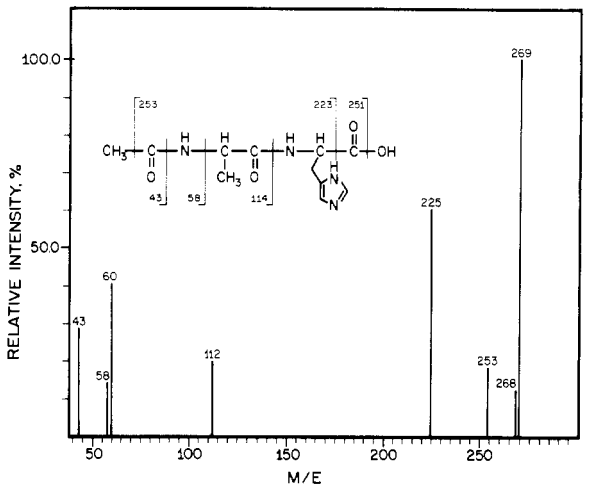

FIGURE 7: Mass spectrum of Ac-Ala-His.

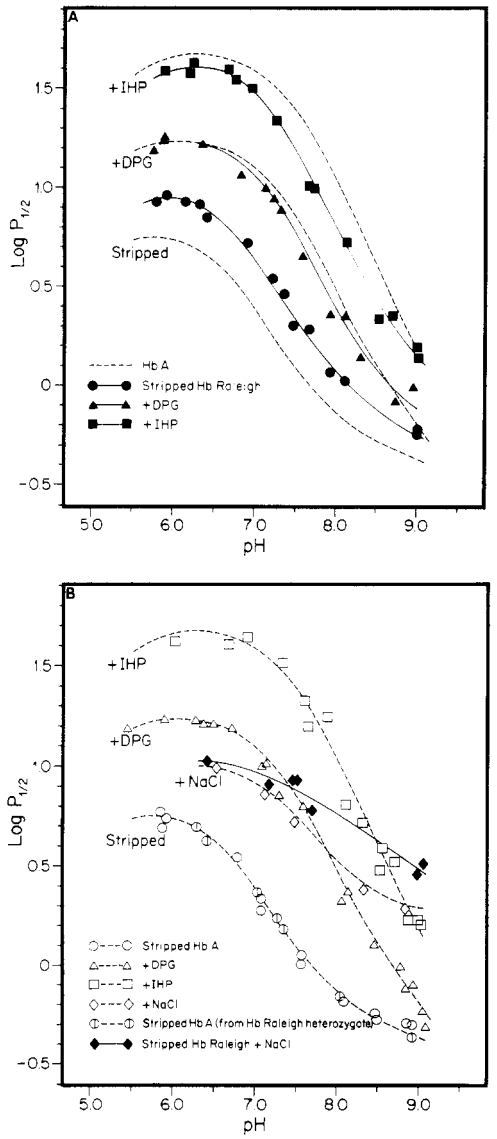

FIGURE 8: (A) The effects of pH and organic phosphate on the oxygen affinity of 50–60 μM solutions of stripped Hb Raleigh at 20 °C in 0.05 M Bistris or Tris buffer. Saturating concentrations (100-fold over tetramer) of organic phosphates were used. Dashed lines show curves obtained under the same conditions with Hb A. (B) The effect of 1 M NaCl on the oxygen affinity of Hb Raleigh and Hb A. Conditions are as in A. Hb A data points are included which show the experimental basis for the dashed lines drawn in A.

TABLE III: Effect of DPG and Ins-P_6 on the Oxygen Affinity of Hb A and Hb Raleigh.[a]

	+DPG		+Ins-P_6	
pH	Hb A	Hb Raleigh	Hb A	Hb Raleigh
6.0	0.50	0.27	0.92	0.65
7.0	0.72	0.42	1.20	0.86
8.0	0.60	0.31	1.16	0.73

[a] These results are expressed as Δ log $P_{1/2}$ values. The data are taken from Figure 8A.

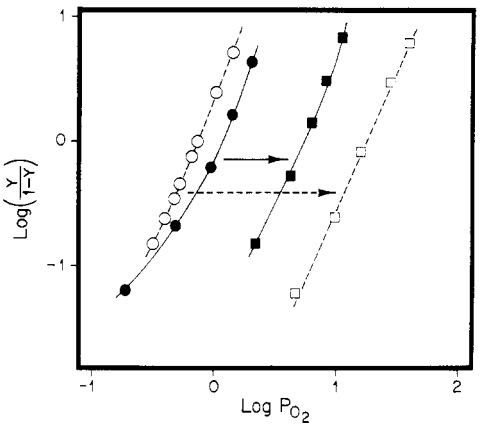

FIGURE 9: Effect of inositol hexaphosphate on Hill plots of oxygen binding by Hb A and Hb Raleigh at pH 8.0 in 0.05 M Tris at 20 °C: (○) stripped Hb A; (□) +Ins-P_6; (●) stripped Hb Raleigh; (■) +Ins-P_6.

which corresponds to the acetyl protons. This is in good agreement with the results of Cozzone and Marchis-Mauren (1970). The resonance upfield from the acetyl peak broadened by partially resolved spin–spin coupling is due to the β-methyl protons of alanine at their usual resonance position (Wüthrich, 1976). Both of these peaks are missing in a spectrum, obtained under identical conditions, of the same peptide from normal hemoglobin.

These results can only be interpreted in terms of the presence of a covalently attached *N*-acetyl group. The equal intensities of the alanine methyl and the acetyl methyl imply all of the peptides present are blocked.

Oxygen Equilibrium Studies. The pH dependence of oxygen binding by stripped Hb Raleigh with and without organic phosphate cofactors is shown in Figure 8A. Stripped Hb Raleigh has a lower oxygen affinity than does stripped Hb A throughout the pH range examined. In addition, DPG and Ins-P_6 have less effect on the oxygen affinity of Hb Raleigh than on that of Hb A. These cofactor effects are summarized in Table III. In contrast, chloride ions have a greater effect on Hb Raleigh than on Hb A (Figure 8B). It is apparent that NaCl acts as an effective modulator of oxygen affinity even when the amino terminus of the β chain is blocked. The significance of these cofactor effects will be discussed in a later section.

Stripped Hb Raleigh is very similar to stripped Hb A in its pH sensitivity. In the presence of organic phosphate cofactors, however, the alkaline Bohr effect appears to be slightly greater for Hb Raleigh than for Hb A, perhaps because of alteration in the pH dependence of organic phosphate binding when the α amino group of the β chain is blocked.

Figure 9 shows oxygen binding curves for Hb Raleigh and Hb A in the presence and absence of the allosteric effector

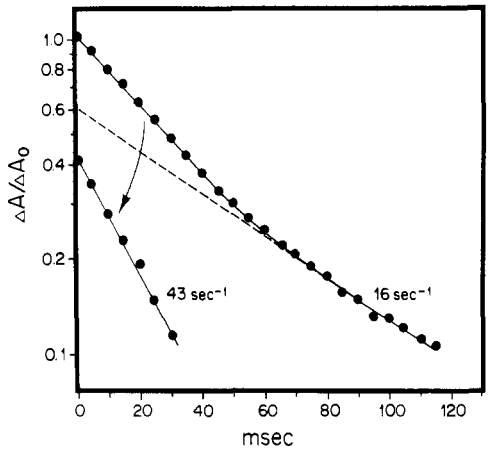

FIGURE 10: Time course of oxygen dissociation from a 10 μM solution of Hb Raleigh in 0.05 M Bistris at pH 7.0, 20 °C (after rapid mixing with a buffer containing sodium dithionite). The dashed line, representing an exponential process with a rate of 16 s^{-1}, was used as a base line for calculating the rate of the faster phase of oxygen dissociation.

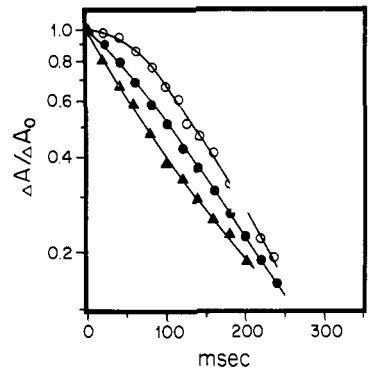

FIGURE 11: The time course of CO combination with a 10 μM solution of Hb Raleigh observed after rapid mixing with a 100 μM solution of CO is shown to have a distinct wavelength dependence. Wavelengths at which observations were made were: (A) 437.5 nm (●); (B) 425 nm (○); and (C) 426 nm (▲). In this experiment Hb Raleigh was in 0.05 M Bistris at pH 7.0, 20 °C.

Ins-P_6. These binding curves show that the degree of cooperativity of oxygen binding measured at 50% saturation is essentially unaffected by the amino acid substitution in Hb Raleigh. At low degrees of saturation, the Hill plots of oxygen binding to Hb Raleigh differ from those for Hb A in that n values are somewhat decreased. This decrease may be ascribed to an increased heterogeneity in the oxygen binding sites or to increased stabilization of the deoxy (T) state. It is significant that Hb A is characterized by asymmetric Hill plots of oxygen binding only in the presence of organic phosphate cofactors.

Oxygen Kinetics. The dissociation of oxygen from Hb Raleigh was studied by observing the spectral change which occurs when an air-equilibrated hemoglobin solution is rapidly mixed with an equal volume of buffer containing sodium dithionite. The time course of oxygen dissociation from Hb Raleigh (Figure 10) differs from that observed for Hb A in that two distinct phases in oxygen dissociation are apparent. At pH 7.0 the initial phase amounts to about 40% of the reaction and has a rate of 43 s^{-1}. The final phase has a rate of about 16 s^{-1}. At this pH a fairly homogeneous time course is observed for Hb A, with a rate of approximately 24 s^{-1}. The biphasic time course observed for Hb Raleigh may be an indication that functional differences between the α and β chains are greater in the Hb Raleigh tetramer than in Hb A.

Carbon Monoxide Kinetics. The kinetics of CO combination were examined by rapid mixing and flash photolysis techniques. When deoxy-Hb Raleigh is rapidly mixed with a buffer containing CO, the time course of CO combination is autocatalytic, with a final rate approximately twice that observed in early stages of the reaction. At pH 7.0, the final phase of CO combination has a second-order combination velocity constant of approximately 3.3×10^5 M^{-1} s^{-1}. This value is slightly higher than the apparent rate constant observed for Hb A under the same conditions, which increases from 1.6 to about 2.5×10^5 M^{-1} s^{-1} during the process of CO binding. A remarkable feature of the CO combination kinetics of Hb Raleigh is that the time course of this variant shows a more pronounced wavelength dependence than that of normal hemoglobin A (Figure 11). The time course observed at 437.5 nm is like that seen at most wavelengths. Differences are apparent at 425 and 426 nm, wavelengths close to the isosbestic point between deoxy and CO hemoglobin. The time course shows a pronounced autocatalytic character at 425 nm, but at 426 nm is more nearly represented by a single exponential process.

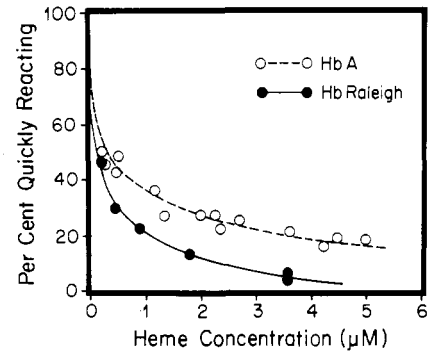

FIGURE 12: Effect of dilution on the percentage of quickly reacting material observed after complete flash photolysis of the CO derivative of Hb A and Hb Raleigh with both proteins in 0.05 M Bistris at pH 7.0, 20 °C (observed at a wavelength of 437.5 nm).

As in the oxygen dissociation and equilibrium experiments, this enhanced heterogeneity and wavelength dependence may be an indication of enhanced α and β chain differences.

In flash photolysis studies of CO binding at neutral pH, some quickly reacting material is apparent that is not seen in rapid mixing experiments. The percentage of the quickly reacting material increases as the heme concentration is decreased, and the extent of dissociation into highly reactive subunits may be estimated from the percentage of the fast phase. Plots of the percentage of the quickly reacting material vs. protein concentration for Hb Raleigh and for Hb A are shown in Figure 12. At a given protein concentration the percentage of the fast phase observed for Hb Raleigh is significantly lower than the percentage observed for Hb A. This implies that the dimer–tetramer equilibrium is affected by the mutation so that liganded Hb Raleigh has a much lower tendency to dissociate into subunits than does Hb A.

Discussion

Hb Raleigh (β1 Val→Ac-Ala) is a naturally occurring human hemoglobin variant which constitutes approximately 45% of the hemoglobin in an individual who is heterozygous for Hb A and Hb Raleigh. Blocked or acetylated human hemoglobins are generally only minor fractions of the total hemoglobin present (Bunn et al., 1975; Schroeder et al., 1962). The study of Hb Raleigh and other N-terminally modified hemoglobins is of interest because the β1 position is involved in the binding of cofactors and in the transport of carbon

dioxide (Arnone, 1972; Kilmartin and Rossi-Bernardi, 1969). Moreover, the β-chain N terminus is not considered to be a Bohr group, although its state of ionization changes as the pH increases from pH 6 to pH 9 (Perutz et al., 1969). This observation is supported by our finding of essentially the same pH dependence of oxygen binding for Hb Raleigh as is found for Hb A.

The results of this study show that DPG and Ins-P_6 interact with Hb Raleigh but that their effectiveness is reduced. This finding can be related to the alteration of the β1 residue which normally contributes directly to the charge cluster involved in the binding of cofactors. It is clear that DPG and Ins-P_6 can be bound even when the β-chain N terminus is not charged. The binding of these cofactors stabilizes the deoxy conformation and shifts the allosteric equilibrium between the high affinity (R) and low affinity (T) states further toward the T state.

Our findings may be compared with those of Bunn et al. (1970) who reported virtually no reactivity of DPG with Hb F_1. In Hb F_1 the amino-terminal residues of the γ chains were acetylated. These properties may be ascribed in part to differences in the organic phosphate binding sites of the β and γ chains. In particular, it should be noted that β143 His (H21) is replaced by an uncharged serine in the primary sequence of the γ chain.

The β1 valines are also a possible binding site for small anions such as chloride and phosphate (Arnone, 1972). Our data show that high chloride concentrations lower the oxygen affinities of Hb A and Hb Raleigh and concomitantly alter the pK of some oxygen-linked groups in such a way as to oppose the normal Bohr effect. However, it is remarkable that the effect of 1 M NaCl on the affinity and pH dependence of O_2 binding is more pronounced for Hb Raleigh than for Hb A. The N terminus of the β chains is clearly not the only site for oxygen-linked binding of inorganic anions. These results are in agreement with those of Nigen and Manning (1975), who studied hemoglobin in which the amino-terminal residues of the β chains were carbamoylated.

Stripped Hb Raleigh has a lower oxygen affinity than stripped Hb A, and this characteristic is consistent with kinetic measurements which indicate that the variant hemoglobin has less tendency to dissociate into dimers. There are no accompanying changes in cooperativity. The lower oxygen affinity is similar to that of other hemoglobins which are blocked at the N terminus of the polypeptide chain (Bunn et al., 1970). Lower oxygen affinities are also characteristic of some human hemoglobin variants with mutations which result in a reduction of positive charge density at the cofactor binding site (Bonaventura et al., 1974a,b, 1976). Reduction in the net positive charge of the DPG binding site by a mutational event could be a factor in decreasing the oxygen affinity through stabilization of the deoxy conformation of the variant hemoglobin. This reduction would provide a possible explanation for the further decrease in oxygen affinity caused by the binding of DPG and NaCl. These anions would effectively reduce the positive charge of the anion binding site and counteract repulsive interactions between the positively charged residues of the binding site. Conversely, an increase in oxygen affinity is observed for variants in which mutation leads to an increase in the positive charge density of the DPG binding site (Bonaventura et al., 1975a,b).

A recent survey of N-terminal acetylated proteins (Jornwall, 1975; Bloemendal, 1977) indicated that proteins with serine, alanine, and glycine at the N terminus account for approximately 77% of these proteins. Furthermore, acetylation (which appears to be a highly specific reaction) occurs at an early posttranslational stage (Marchis-Mouren and Lipmann, 1965) and is favored by the presence of a high percentage of branched-chain and charged residues among the first ten amino acids. These conditions are met by the β chain of Hb Raleigh and the γ chain of Hb F_1. It is therefore quite probable that acetylation of the amino-terminal residues of Hb Raleigh might be a consequence of the substitution of alanine for valine.

Attempts to acetylate Hb A with aspirin have been reported, but in all cases acetylation involved the ϵ-amino group of lysine (Bridges et al., 1975; Shamsuddin et al., 1974). Although the structural and functional significance of N-terminal acetylation is unclear, in Hb Raleigh, acetylation alters the allosteric site where organic phosphates are bound and clearly leads to alterations in the hemoglobin's functional properties.

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Effect of Pressure upon the Fluorescence of Various Flavodoxins†

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ABSTRACT: The effects of hydrostatic pressure in the range of 10^{-3} to 11 kbar on the fluorescence of flavodoxins from *Peptostreptococcus elsdenii, Desulfovibrio vulgaris, Azotobacter vinelandii,* and *Clostridium* MP were investigated. The first three flavoproteins showed under high pressure enhancements of flavin fluorescence of over 50 times resulting from the release of flavin mononucleotide from the protein complex. The Clostridial flavodoxin showed a very much smaller fluorescence change. At pH 7.5 the high-pressure fluorescence changes of the flavodoxins of *D. vulgaris* and *P. elsdenii* were not reversed by decompression, but in *A. vinelandii* the pressure changes were over 80% reversible. At pH 5 over 80% reversibility was restored to the flavodoxins of *D. vulgaris* and *P. elsdenii*, although the pressure dependence of the fluorescence changes was very similar in the reversible and irreversible cases. The midpoint pressures in the reversible reactions were 4.7 kbar (*D. vulgaris*), 8.7 kbar (*P. elsdenii*), and 10.6 kbar (*A. vinelandii*) indicating specific differences in the flavin binding regions. Apparent volume changes in these reactions were 65–75 mL/mol indicating participation of a large fraction of the protein in the pressure-induced changes. The irreversible changes are not related to protein aggregation and are believed to result from a pressure-dependent covalent modification, not yet characterized, of the flavin binding region of the protein.

Fluorescence techniques can be conveniently applied to study denaturation processes in proteins by means of hydrostatic pressure (Li et al., 1976a,b). Following the investigation on the effect of pressure upon the flavin-binding protein (Li et al., 1976a) we have investigated the effects of high pressure upon another class of flavoproteins, the flavodoxins. Flavodoxins are single peptide-chain proteins of relatively low molecular weight (15 000–23 000) which function as low-potential electron carriers in a variety of organisms (Knight et al., 1966; Shethna et al., 1966; Mayhew and Massey, 1969; Dubourdieu and LeGall, 1970; Mayhew, 1971a). The flavodoxins contain a single, noncovalently attached molecule of flavin mononucleotide (FMN) per molecule of protein. From the large flavodoxin family, four of them isolated from different bacterial strains were selected for this study, namely flavodoxin from *Peptostreptococcus elsdenii, Clostridium* MP, *Desulfovibrio vulgaris,* and *Azotobacter vinelandii.* These flavodoxins are well characterized. The amino acid sequences of the first three are known (Tanaka et al., 1973, 1974; Dubourdieu et al.,

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