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Assignment of Fluorine Nuclear Magnetic Resonance Signals from Rabbit Cyanomethemoglobin†

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ABSTRACT: A fluorine NMR study of cyanomethemoglobin prepared from hemoglobin isolated from rabbits maintained on a diet containing DL-*p*-fluorophenylalanine is described. The results indicate that substitution of fluorophenylalanine occurs essentially randomly at all phenylalanine positions of the α- and β-globin chains; a set of hybrid hemoglobins in which only the α- or only the β-chains contain the fluorinated amino acid was prepared and used to ascertain the fluorine NMR signals arising from each chain. The temperature and pH dependences of chemical shifts, spin-lattice relaxation times, $^{19}F\{^1H\}$ nuclear Overhauser effects, and the effect of chemical modification of the β-93 sulfhydryl groups were examined. When considered in light of presently available X-ray structures of human and horse hemoglobins, the available data permit a tentative assignment of most signals to particular fluorophenylalanine/phenylalanine positions in the globin sequences.

Fluorine nuclear magnetic resonance (NMR)[1] spectroscopy is a powerful tool for examining biological structures because of the high sensitivity of fluorine chemical shifts to the details of local molecular structure. Many organisms will incorporate fluorinated amino acids present in their growth medium or diet into proteins (Sykes & Weiner, 1980), and these proteins often exhibit a number of resolved resonances in their fluorine NMR spectra. In these cases, as in all high-resolution NMR spectra, maximum structural information is obtained when the observed signals can be assigned to particular molecular features, that is, when each fluorine resonance can be associated with a particular amino acid of the protein sequence. Assignments of the fluorine signals from 3-fluorotyrosine-labeled *lac* repressor have been made (Jarema et al., 1981), but beyond this elegant work, there have been few attempts to establish the correspondence between a particular fluorine resonance and a particular amino acid position in a protein which contains biosynthetically incorporated fluorinated amino acids.

Westhead & Boyer (1961) have reported that DL-*p*-fluorophenylalanine replaces a significant fraction of phenylalanine in proteins of the rabbit when the animal is maintained on a diet containing this fluoro amino acid. We have examined the hemoglobin formed by these animals when 0.3% (by mass) of fluorophenylalanine is present in the diet. Under these conditions about one replacement of phenylalanine per $\alpha_2\beta_2$ tetramer takes place, and the fluorine NMR spectrum of the cyanomet form of the protein is consistent with the presence of 16 resonances, one for each phenylalanine/fluorophenylalanine position in the sequences of rabbit α- and β-globins. In attempting to assign these signals to particular positions, the genetic approach of Jarema et al. (1981) is not yet open to us, and assignments based on a collection of observations including temperature and pH variation of chemical shifts, spin-labeling, spin-lattice and spin-spin relaxation rates, and fluorine-proton nuclear Overhauser effects are reported. We emphasize, at the outset, that the assignments are tentative and rest on a presumed close homology between the X-ray crystal structures of human or horse hemoglobins and the structure of rabbit hemoglobin in solution. The cyanomet form

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; ^1H NMR, proton NMR; UV-vis, ultraviolet-visible; NOE, nuclear Overhauser effect; CSA, chemical shift anisotropy; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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of this hemoglobin was chosen for initial study because this molecule can withstand broader variations in pH and temperature than other ferric or ferrous hemoglobin derivatives (Antonini & Brunori, 1971).

Experimental Procedures

Materials. Deuterium oxide (99.8%) was obtained from Stohler Isotope Chemicals. Inorganic salts were Mallinckrodt AR grade. Tris buffer was purchased from Sigma, DL-*p*-fluorophenylalanine was obtained from Calbiochem while α-fluorocinnamic acid was provided by Aldrich Chemical Co. L-*p*-Fluorophenylalanine was prepared from the racemic material according to the procedure of Tong et al. (1971). *N*-(2,2,6,6-Tetramethyl-4-piperidinyl-1-oxy)iodoacetamide (I) was prepared as described by McConnell et al. (1969) with reagents supplied by Aldrich. *N*-(2,2,6,6-Tetramethylpiperidinyl)iodoacetamide (compound II) was prepared by treating 4.9 mL (0.029 mol) of 4-amino-2,2,6,6-tetramethylpiperidine (Aldrich) and 4 mL of triethanolamine dissolved in 50 mL of toluene with a solution of 2.3 mL of chloroacetyl chloride in 10 mL of toluene, added dropwise over the course of 1 h. The reaction was stirred rapidly during the time of the addition and then for another hour. The reaction mixture was extracted with five 20-mL aliquots of 1 N HCl, and the combined aqueous extracts were neutralized with 6 N NaOH until precipitation ceased (pH 12–13). The precipitate was collected by filtration, washed with 10 mL of 1 N NaOH, and dried in vacuo. This material had a proton NMR spectrum in complete accord with the expected structure. The compound was converted to the iodide by treatment with KI in anhydrous acetone following the procedure of McConnell et al. (1969). The NMR spectrum was distinct from that of the chloride and in accord with the expected structure.

Carboxypeptidases A and B, treated with diisopropyl fluorophosphate to minimize tryptic and chymotryptic activity, were purchased from Sigma.

Animal Husbandry. New Zealand white rabbits (*Oryctalagus cuniculus*) were obtained from local rabbitries at approximately age 2 weeks and housed in the campus Central Vivarium under approved conditions. Water was available ad libitum. Purina Checkers chow was used as received or spiked with 0.3% fluorophenylalanine by mass according to three procedures. (1) A powder was obtained by grinding the chow and amino acid in a Waring blender. (2) Biscuits were formed from a slurry of ground chow pellets and a solution of fluorophenylalanine in water by pressing the mixture into forms that had a semicircular cross section and then drying in an oven at 150 °C. (3) A mixture of intact chow pellets and dry amino acid was rotated slowly in a sealed jar until the amino acid coated the outside of each pellet. At the low amounts of fluorophenylalanine used in the present study the last procedure was most convenient and afforded a product that was vastly preferred by the animals.

Hemoglobin Isolation. Blood was drawn at approximately biweekly intervals by venipuncture of the ear. Samples ranging from 25 to 40 mL were collected in Oak Ridge type centrifuge tubes containing 1.4 mL of 25% sodium citrate (w/v). With all subsequent operations carried out at 4 °C, the red cells were pelleted at 4000*g* in a RC2-B centrifuge fitted with a SS-34 rotor for 10 min. After removal of the serum and buffy layer, the cells were suspended in approximately 25 mL of 1% NaCl solution and repelleted. This washing procedure was repeated a total of 3 times. The cells were then lysed by adding 6–10 volumes of H_2O and gently stirring for 2 h. The hemolyzate was centrifuged at 20000*g* for 1 h, and the supernatant was transferred to an Amicon ultrafiltration cell and concentrated to 25 mL by filtration through a PM-10 or PM-30 membrane. For isolation of the cyanomet form of the protein a 20% excess of the stoichiometric amount of $K_3Fe(CN)_6$ was then added to the sample. After 5 min, the hemolyzate was applied to a 2.5 × 100 cm column of Sephadex G-25 (medium; Pharmacia). The hemoglobin-containing band was eluted with 0.1 M Tris buffer, 0.1 M NaCl, and 5×10^{-5} M EDTA, pH 7.9, in order to remove organic phosphates (Berman et al., 1971; Sharma et al., 1980) and ferro- and ferricyanide. Phosphate determination by the method of Ames & Dubin (1960) shows that a minimum of 99% of the endogenous phosphate present in the hemolyzate is removed by this procedure. The hemolyzate was dialyzed against a total of 4 L of solution that was 0.1 M in Tris buffer, 0.1 M in NaCl, 5×10^{-5} M in EDTA, and 0.01 M in KCN at pH 7.5. After dialysis, the solution was concentrated to 10 mL or less by ultrafiltration through a PM-30 membrane. The concentration of protein was determined by diluting an aliquot of this solution and measuring the absorbance at 540 nm; an extinction coefficient of 10.12 mM^{-1} cm^{-1} was used. This extinction coefficient was determined with hemoglobin from controls by the pyridine hemochrome method (Waterman, 1978; Falk, 1964), using an extinction coefficient for the reduced pyridine hemochrome of 34.3 mM^{-1} cm^{-1} at 557 nm.

Chromatography of fluorine-containing cyanomethemoglobin on Whatman CM-32 carboxymethylcellulose according to the procedure of Schroeder & Huisman (1980) produced a single symmetrical band in the elution profile when monitored at 435 nm with an ISCO UA-5 monitor. Chromatography on DEAE-cellulose or chromatography of the apoprotein (see below) provided no evidence of protein heterogeneity. The material also appeared homogeneous when electrophoresed according to the procedure of Riggs (1981). Isoelectric focusing of this material on Ampholine plates (LKB) produced a major band at p*I* = 6.4 and several minor bands (p*I* = 6.0–6.3), as did hemoglobin from control animals.

Amino Acid Analysis. A 300-μL aliquot of hemoglobin solution was applied to a 1.5 × 30 cm column of Sephadex G-25 and eluted with water to remove salts. Concentration of the eluant was determined by the pyridine hemochrome method, and sufficient sample to provide 60 μmol of protein was transferred to a hydrolysis vial. After the sample was dried overnight in a vacuum desiccator, 2 mL of constant boiling HCl was added, and the samples were subjected to at least three freeze–thaw degassing cycles before being sealed with a torch. Hydrolysis at 110 °C for 24 h was followed by amino acid analysis on a Beckman 120 C analyzer with ninhydrin detection. By use of control samples, *p*-fluorophenylalanine was found to elute with base-line resolution following phenylalanine when the standard two column and sodium citrate buffer system was used.

Carboxypeptidase Digestion. The procedure of Antonini et al. (1961) was followed except that boiling the solution was used to precipitate protein and halt the reaction. The progress of the digestion was followed by removing aliquots and testing for the presence of histidine and tyrosine by paper chromatography; digestion was halted when the amounts of these amino acids produced by the reaction appeared to be equal.

Preparation of Hemoglobin Hybrids. Preparation of hybrid rabbit hemoglobins containing fluorinated α-chains or fluorinated β-chains followed a procedure similar to that of Dintzis (1961). Solutions of normal and fluorinated hemoglobins were dialyzed against distilled water at 4 °C. The concentration of protein in each solution was adjusted to 25 mg/mL. The

following operations were then carried out in parallel for each. Hemin was removed by adding 40 mL of solution dropwise to a mixture of 900 mL of acetone and 0.45 mL of concentrated HCl at −20 °C. Most of the acetone was decanted, and the precipitated globins were collected by centrifugation. A second 40-mL aliquot was similarly treated and the pelleted protein from both extractions combined; the combined pellets were then dissolved in 60 mL of 0.2 M formic acid and 0.02 M pyridine solution. The mixture was vigorously stirred until the globins had redissolved. The solution was applied to a 2.5 × 50 cm column of Whatman CM-32 carboxymethylcellulose that had been preequilibrated with 0.2 M formic acid and 0.02 M pyridine solution. Elution with a concentration gradient of the pyridine–formate buffer from the initial conditions to concentrations 10 times the initial concentrations was started; 5-mL fractions were collected and the elution followed at 280 nm with an ISCO Model UA-5 absorbance monitor. α-Globin eluted first, in agreement with observations of Dintzis, and was recognizable by its smaller extinction at 280 nm relative to the extinction of the β-globin. The chromatograms showed only single bands for the α- and β-globins, and there was no indication of heterogeneity in either material. Fractions corresponding to α-globin were combined and concentrated by ultrafiltration on a PM-10 membrane. β-Globin fractions were similarly concentrated. Samples of the globins were dialyzed overnight against 0.01 M potassium phosphate buffer, pH 7.5. The absorbance of each protein solution was determined at 280 nm, and when the extinction coefficients of Yip et al. (1972) for human globins were used, the appropriate amounts of fluorinated and non-fluorinated chains were combined to give a 1:1 stoichiometry. The globin mixtures were dialyzed against 0.01 M potassium phosphate buffer (pH 7) at 4 °C overnight or until the pH of the protein solution reached 6.9. A significant amount of protein precipitates during the dialysis. To each recombined globin solution at 4 °C was added dropwise 50 mL of bovine hemin (1.6 mg/mL in 0.01 M K_3PO_4 and 0.01 M NaCl adjusted to pH 7), and, after stirring for 30 min, precipitated material was removed by centrifugation (20000*g*, 15 min). The supernatants were concentrated to 10–15 mL by ultrafiltration (PM-10 membrane) and then applied to a 1.5 × 30 cm Sephadex G-25 column equilibrated with 0.02 M K_3PO_4 and 0.01 M KCN at pH 6.8. Elution with the same buffer followed. The major band, contained in approximately 20 mL, was collected and applied to a 1.5 × 30 cm column of CM-Trisacryl M (LKB). The column was eluted with the same buffer, and after the rapidly moving initial band (shown to be CN-methemin by visible spectroscopy) had emerged, the eluent was changed to 0.05 M K_3PO_4 and 0.01 M KCN, at pH 8. Hemin-free, recombined cyanomethemoglobin then eluted from the column within 50 mL. Finally, each hybrid hemoglobin was applied to a 2.5 × 100 cm column of Sephadex G-25, equilibrated with 0.1 M Tris and 0.1 M NaCl, p 7.9, and eluted with the same buffer in order to remove phosphates. Concentration by ultrafiltration was used to provide samples for NMR studies. Visible spectra and electrophoretic and isoelectric focusing patterns for these hybrids were essentially identical with those of native cyanomethemoglobin from controls.

Spin-Labeled Hemoglobin. Hemoglobin with a spin-label present at the sulfhydryl group of cysteine β-93 was prepared by treating 7.5 mL of a solution of fluorinated rabbit hemoglobin (137 mg/mL) in a buffer composed of 0.1 M Tris, 0.1 M NaCl, and 0.01 M KCN at pH 7.5 with 20 mg of sodium dithionite. The mixture was immediately applied to a 1.5 × 30 cm column of Sephadex G-25 that had been equilibrated with 0.05 M K_2HPO_4 solution at pH 7.8 and eluted with the same buffer. The visible spectrum of the eluate showed a ratio of absorbances at 577 and 540 nm consistent with the presence of oxyhemoglobin. The eluted protein was diluted to a total volume of 10 mL with phosphate buffer and, at 4 °C, treated with 43 mg of *N*-(3,3,6,6-tetramethyl-4-piperidinyl-1-oxy)-iodoacetamide. (Some spin-labeling agent was insoluble but served to keep the solution saturated.) After 48 h, a small aliquot was removed, and to the remaining solution was added 10 mg of potassium ferricyanide. The mixture was applied to a 2.5 × 10 cm column of Sephadex G-25 equilibrated with 0.1 M Tris, 0.1 M NaCl, and 0.01 M KCN, pH 7.9, and eluted with the same buffer. The eluted material was dialyzed overnight against 4 L of 0.1 M Tris, 0.1 M NaCl, and 0.01 M KCN at pH 7.5 and then concentrated to about 6 mL by ultrafiltration (PM-30 membrane). Sufficient D_2O (5%) to provide an NMR lock signal was added. Sulfhydryl group titrations using Ellman's reagent (Geraci et al., 1969) typically showed 1.6–1.9 SH groups per tetramer. Titrations performed by using (hydroxymercuri)benzoic acid (Benesch et al., 1965) gave results in the same range. After reaction with the spin-labeling reagent, the sulfhydryl titer was reduced to approximately 0.4 SH group per tetramer. Exposure to the reaction conditions for an additional 48-h period did not alter this titer.

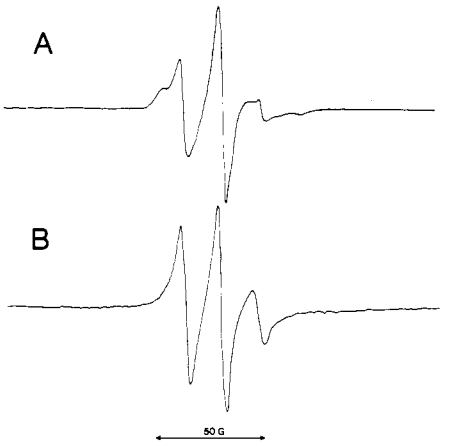


FIGURE 1: ESR spectra of spin-labeled oxy (top) and cyanomet (bottom) fluorophenylalanine-containing rabbit hemoglobin. The spectra were obtained with a Varian E-4 instrument using samples contained in glass capillaries.

The ESR spectrum of the spin-labeled protein as the oxy form is very similar to the spectrum exhibited by human hemoglobin treated with the same agent (McConnell, 1971); the ESR spectrum of the oxy and cyanomet forms of the protein are shown in Figure 1. ESR experiments showed that treatment with sodium dithionite quickly reduced the spin-label on the protein to a species with no ESR signals above background.

Hemoglobin modified with *N*-(2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (II) was obtained by a procedure similar to that used for preparation of the spin-labeled material.

Procedures. Sample pH was determined with a Radiometer PHM52 meter fitted with a Radiometer microcombination electrode. For samples containing deuterium oxide the meter reading is reported as "pH", and no corrections are made for the deuterium content of the solvent.

UV–vis spectroscopy was carried out with a Cary Model 15 spectrophotometer at ambient temperature by using matched 1-cm cells.

Fluorine and proton NMR spectroscopy employed a Nicolet NT-300 instrument operating at 282 or 300 MHz, respectively.

The fluorine probe (10 mm) was designed to have a negligible fluorine background signal and to permit $^{19}F\{^1H\}$ double resonance experiments. Sample temperatures were maintained with the Nicolet controller and were calibrated by using the peak separation in the proton spectrum of methanol (Van Geet, 1970; Raiford et al., 1979). Fluorine spectra were referenced to the signal provided by a capillary containing solutions of α-fluorocinammic acid or 5-fluorosalicylic acid (2 mg/mL in methanol). *p*-Fluorophenylalanine was found to have its resonance 9.73 ppm downfield of the first reference or 10.54 ppm downfield of the second.

Samples for fluorine NMR spectroscopy were typically 1–3 mM in hemoglobin, contained in a solvent consisting of 5% deuterium oxide in water with 0.1 M Tris, 0.1 M NaCl, and 0.01 M KCN present at pH 7.5. Samples were usually examined within 2 weeks of preparation, but storage of solutions at 4 °C for over 1 year did not alter the observed spectra. For pH-variation experiments a sample of cyanomethemoglobin in 0.1 M Tris buffer containing 0.1 M NaCl and 0.01 M KCN at pH 7.5 was divided in half. One portion was dialyzed against 0.01 M Tris, 0.1 M NaCl, and 0.01 M KCN at pH 9 while the other was dialyzed against 0.1 M Bis-Tris and 0.1 M NaCl at pH 6.0. The two fractions were concentrated to equal volumes, and sufficient D_2O was added to give a 5% D_2O solution. Samples at intermediate pH values were obtained by mixing appropriate volumes of the two solutions. A second set of solutions at pH 5 and pH 6 were prepared with only Bis-Tris buffer to cover the pH range 5–6. pH of the NMR samples was measured before and after the fluorine spectrum was obtained and showed no change; pH-induced effects in the spectra appeared to be reversible.

Typically, spectra were obtained with a 20 000 Hz sweep width represented by 8K data points, giving a digital resolution of 0.009 ppm. Free-induction decays were multiplied by an exponential function sufficient to generate line broadenings of 25–40 Hz. All spectra were collected under conditions where a delay of at least $5T_1$ was observed between acquisitions.

Fluorine spectra at 188 MHz were obtained on a Nicolet NT-200 under similar conditions.

Results

Extent of p-Fluorophenylalanine Incorporation. Hemoglobin isolated from rabbits fed chow containing *p*-fluorophenylalanine appeared to be homogeneous upon chromatography on ion-exchange columns under conditions which are adequate for resolution of the various forms of human hemoglobin (Schroeder & Huisman, 1980). The materials also gave a single band when electrophoresed in polyacrylamide gels although isoelectric focusing of the cyanomet proteins on LKB Ampholine plates revealed the presence of several minor fractions in both normal and fluorine-containing proteins.

Amino acid analyses of hemoglobin from a control animal and two animals (R3 and R6) maintained on a chow containing 0.3% DL-*p*-fluorophenylalanine were carried out and compared to expectations based on the published sequence for rabbit hemoglobin (Braunitzer et al., 1966; Flamm et al., 1971). Except for lysine, alanine, valine, and leucine, the observed composition agreed well with the sequence data (Table I). Small amounts of *p*-fluorophenylalanine were detected in the hemoglobins from animals receiving this amino acid in their diet. Analysis of hemoglobin from an animal receiving a diet containing 0.15% of the L isomer showed that significantly less fluorophenylalanine incorporation had taken place in this case, 0.5 ± 0.1 mol of this amino acid being found per mol of protein.

Table I: Amino Acid Analysis

amino acid	control animal	mol/mol of hemoglobin R3[a]	R6[a,b]	theory[c]
Lys	43.0	44.3	43.5, 41.5	48
His	36.8	36.3	41.6, 37.0	40
ammonia	27.6	32.4	33.5, 29.6	38–42
Arg	11.4	11.6	12.1, 11.3	12
Asp	44.2	45.9	43.4, 43.7	46
Thr	30.0	31.9	31.4, 30.7	30–32
Ser	36.7	38.4	36.2, 36.7	42–44
Glu	43.7	44.7	42.3, 42.2	42–46
Pro	22.3	22.3	21.4, 22.1	22
Gly	39.1	40.8	39.5, 38.5	40
Ala	53.1	56.2	51.9, 52.8	56
Cys	3.8	3.8	3.9, 3.6	4
Val	46.7	50.6	49.4, 46.4	46
Met	4.4	4.4	4.3, 4.4	4
Ilu	6.1	6.8	6.3, 6.3	8
Leu	62.7	66.3	59.9, 61.3	70–72
Tyr	12.0	12.4	12.0, 11.3	12
Phe	29.9	31.4	29.7, 29.2	30–32
F-Phe		0.8	0.6, 1.1	

[a] Animal received 0.3% DL-*p*-fluorophenylalanine in its diet. [b] Two samples of hemoglobin obtained in bleedings 2 months apart were analyzed. [c] Computed from the data of Braunitzer et al. (1966) and Flamm et al. (1971).

Extent of Tyrosine Replacement. The C-terminal sequences of rabbit α and β-globins are ---Lys-Tyr-Arg-COOH and ---Lys-Tyr-His-COOH, respectively (Flamm et al., 1971; Braunitzer et al., 1966). Digestion of the fluorinated rabbit hemoglobin with carboxypeptidase provided samples in which tyrosine from the penultimate position of the sequences were made available. Amino acid analysis of these digests showed that the amount of *p*-fluorophenylalanine present was 0.035% of the amount of tyrosine. However, a small amount of phenylalanine was also observed in the digest, and if it is assumed that some nonspecific reaction has produced phenylalanine and *p*-fluorophenylalanine and that the ratio of these amino acids is the same as that reported in Table I, one concludes that no *p*-fluorophenylalanine is present at the tyrosine positions. Our results thus suggest that no more than 0.035% replacement of tyrosine by fluorophenylalanine has taken place and probably *no* such substitution has occurred. A similar conclusion was reached by Westhead & Boyer (1961).

If appreciable tyrosine substitution is ruled out, then the fluorophenylalanine in the rabbit hemoglobin isolated has probably replaced phenylalanine. In animals receiving the DL mixture 2.7 ± 0.6% of the phenylalanine positions are so substituted while in those receiving the resolved L-amino acid 1.5 ± 0.2% substitution takes place.

Preliminary Fluorine NMR Observations. Figures 2 and 3 display fluorine NMR spectra of cyanomethemoglobin obtained from rabbits maintained on the fluorophenylalanine-enriched diet.

An identification of those NMR signals which arise from residues of the β-globin was made by preparing hybrid hemoglobins in which the α-chain was from a fluorinated rabbit and the β-chain was from a control animal and vice versa. The reconstituted hybrids were obtainable only in low yield, and it is evident from the fluorine NMR spectra of these materials (Figure 3) that completely native samples probably have not been obtained. However, the spectra were reproducible from preparation to preparation and were independent of magnetic field strength (experiments at 188 and 282 MHz). One can assign the resonances observed with the cyanomet protein as indicated in Figure 3, with the signals labeled A arising from

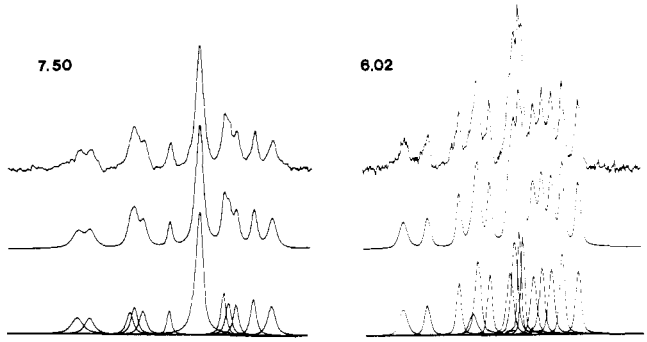


FIGURE 2: Analysis of the fluorine NMR spectrum of *p*-fluorophenylalanine-containing rabbit cyanomethemoglobin at pH 7.50 and 6.02. In each case the top curve is the experimental curve, while the middle trace is a simulation of this curve obtained by summing the Lorentzian functions shown at the bottom.

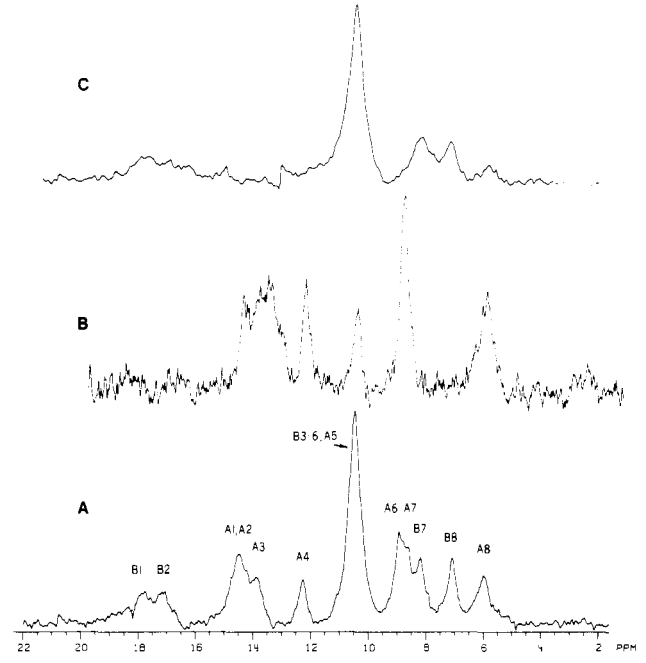


FIGURE 3: Assignment of fluorine resonances to globin chains. (A) Native fluorinated cyanomethemoglobin. (B) Hybrid hemoglobin prepared from fluorine-containing α-globin and native β-globin. (C) Hybrid hemoglobin prepared from native α-globin and fluorinated β-globin. All spectra were obtained at pH 7.5 with solutions containing 0.1 M Tris, 0.1 M NaCl, 0.01 M KCN, and 5% deuterium oxide. The shift axis gives the approximate chemical shift relative to the signal of 5-fluorosalicylic acid in methanol (contained in an external capillary).

the α-globin chains and those designated B due to the β-globins.

For a better indication of the relative intensities of the resonances observed than would be obtained by simple integration of the broad, overlapping peaks, spectra were simulated by summing Lorentzian curves and adjusting the position, width, and height of each function until good agreement between the line shape thus computed and an experimental spectrum was obtained (Figure 2). Within the reproducibility of this process (0.1 residue) all fluorophenylalanine resonances from the α- and β-chains appear to have nearly identical intensities at pH 7.5 with the exception of resonance A4 (Table II). The reason for the reduced intensity of this signal is not clear although we currently believe that the remainder of the intensity for this resonance is buried under the A5, B3–6 combination. It thus appears that substitution of *p*-fluorophenylalanine for phenylalanine in rabbit hemoglobin has taken place randomly, with the extent of substitution being very similar for all residues in both the α- and β-chains.

Table II: Intensities of Fluorine Resonances[a]

signal	relative intensity at pH 7.5	relative intensity at pH 6.0
B1[b]	1.0	0.6[c]
B2[b]	0.8	0.5[c]
A1	0.8	0.7
A2	1.0	1.7
A3	0.7	
A4	0.6	0.7
A5, B3–6	5.2	4.6
A6	1.2	0.9
A7	1.0	0.9
B7	1.0	1.0
B8	1.2	1.1
A8[b]	1.3	0.9

[a] Relative intensities were obtained by line-shape simulation by using the Nicolet program NMRCAP, part of the operating software of the NMR spectrometer. Reproducibility of replicate simulations was about 0.1 residue. [b] These resonances are very broad with poorer signal-to-noise than others so that curve fitting is open to more interpretation and error. [c] The loss of signal intensity is real and is observed in spectra of samples at pH 6 or lower.

Fluorine NMR spectra obtained with hemoglobin samples from animals which received only the L isomer in their diet were similar to those observed with hemoglobin from rabbits maintained on the racemic mixture.

When the protein is exposed to denaturing conditions, the fluorine spectrum is a single line at essentially the same chemical shift that is observed for free *p*-fluorophenylalanine; the 12-ppm span of chemical shifts observed with the native protein must therefore represent the effects of the tertiary structure of the protein.

pH Variation. At constant temperature the chemical shifts of the signals from the fluorinated cyanomethemoglobin are dependent on solution pH. Over the pH range made available by the stability of the protein several, resonances (A2, A3, A6, A7, A8, B1, B2, B7, and B8) exhibited titration behavior of their shifts that could be represented by a single ionization event:

$$AH^+ \overset{K_1}{\rightleftharpoons} A + H^+ \qquad (1)$$

For these cases the data were fit by a nonlinear least-squares method (Conway et al., 1970) to the eq 2, where δ_{obsd} is the

$$\delta_{obsd} = \frac{\delta_{AH}[H^+]}{K_1 + [H^+]} + \frac{\delta_A K}{K_1 + [H^+]} \qquad (2)$$

observed chemical shift, δ_{AH} is the shift characteristic of the protonated form, δ_A is the shift of the ionized form, and K_1 is the equilibrium constant for the ionization. The use of eq 2 rests on the assumption of rapid proton exchange between AH^+ and A. The chemical shift for the fully ionized form (δ_A), the apparent pK_a governing the ionization, and the shift change upon protonation ($\delta_{AH} - \delta_A$) for each resonance which appeared to follow eq 2 are given in Table III.

The pH dependence of the other signals was more complex, indicating that the chemical shifts in these instances were being governed by two (or more) ionizations. Data for these residues did not cover a large range of shifts and were analyzed in terms of a two-step ionization model:

$$AH_2 \overset{K_2}{\rightleftharpoons} AH + H^+ \overset{K_1}{\rightleftharpoons} A + 2H^+ \qquad (3)$$

The data were fit to eq 4 where δ_{AH_2} is the chemical shift of the doubly protonated form, K_2 is the ionization constant for

$$\delta_{obsd} = \frac{\delta_{AH_2}[H^+]^2}{D} + \frac{\delta_{AH_1}K_1[H^+]}{D} + \frac{\delta_A K_1 K_2}{D} \qquad (4)$$
$$D = [H^+]^2 + K_2[H^+] + K_1K_2$$

the first ionization step of eq 3, and the other symbols have the meanings given earlier. The parameters obtained in the fitting exercise are given in Table III. Titration curves are shown in Figure 4; the solid lines are computed by using the data of Table III.

Temperature Effects. Cyanomethemoglobin is paramagnetic, and in such species, nuclei close to the unpaired spin(s) may have their chemical shifts altered by the pseudocontact interaction (Jardetzky & Roberts, 1981). Shifts so affected are expected to depend strongly on temperature. The resonance positions of several signals in the fluorine spectrum observed at pH 7.5 were found to change smoothly with sample temperature over the range 0–35 °C. The slopes of plots of chemical shift vs. the reciprocal of the sample temperature are given in Table IV. Generally, those resonances at highest and lowest field were found to be most temperature sensitive, indicating that the pseudocontact interaction contributes to the extremes of chemical shift observed and showing that some fluorine nuclei must be quite close to the heme rings in the structure.

Spin–Lattice Relaxation and $^{19}F\{^1H\}$ Nuclear Overhauser Effects. Spin–lattice relaxation in the fluorinated hemoglobin was followed by the inversion–recovery method (Becker, 1980). The recovery curves were exponential within the errors of the experiment and were analyzed by using the Nicolet software to provide an apparent spin–lattice relaxation time (T_1) for each resolved resonance. These values are given in Table III.

The line-shape simulations (Figure 2) provide an estimate of the line width of each resonance at half-maximum ($W_{1/2}$). When the contribution to the line width due to H–F spin coupling is neglected, these observed line widths provide an estimate of the transverse relaxation time (T_2) for each resonance according to

$$T_2 = (1/\pi)W_{1/2} \qquad (5)$$

Values for T_2 calculated in this way are given in Table III; all line widths used were corrected for the broadening for signal-to-noise enhancement introduced during workup of free-induction decays.

The fluorine signal intensity for each resonance was substantially reduced by broad-band irradiation of protons. The Overhauser effect (η) was computed for each by using

$$\eta = \frac{F_z - F_o}{F_o} \qquad (6)$$

where F_z represents the integrated signal intensity with proton irradiation while F_o is the signal strength without irradiation. There was no discernible difference in the signal line widths during irradiation due to proton decoupling. This observation helps validate our assumption that H–F couplings do not contribute greatly to the observed line widths. However, the signal-to-noise ratio in the spectra obtained with irradiation is poorer, and small changes in line width would be difficult to detect reliably.

The T_1 and NOE values are estimated to be reliable to within ±10% while the T_2 parameters are less accurate and have an estimated error of ±15%. Overall, the relaxation data for the majority of the resonances are quite similar; those resonances that are strongly pseudocontact shifted have more efficient spin–lattice and spin–spin relaxation processes, as well as a diminished Overhauser effect.

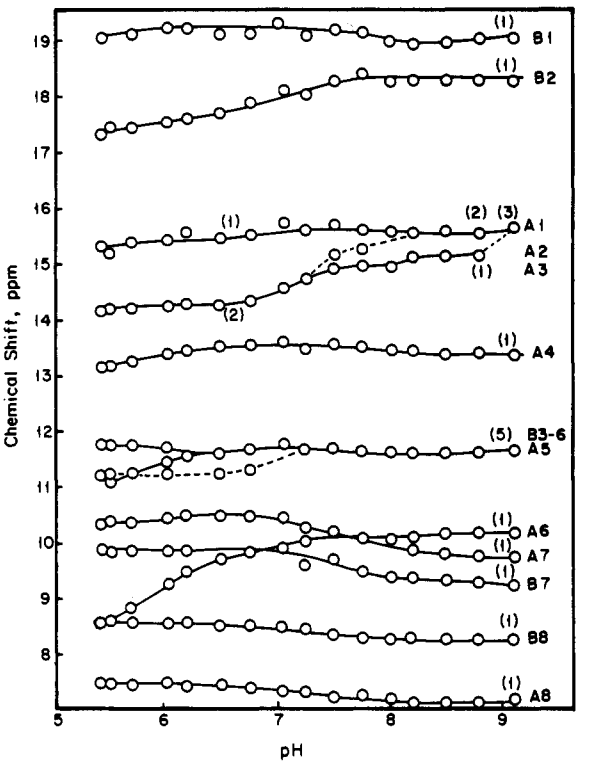


FIGURE 4: Variations with pH of the fluorine NMR signals of cyanomethemoglobin. The solid curves were computed as described in the text.

Relaxation of fluorine nuclei in this protein will involve proton–fluorine dipolar interactions, interaction with the unpaired electron spin of the iron, and the anisotropy of the chemical fluorine shifts. The observed spin–lattice relaxation rate ($R_1^{obsd} \equiv 1/T_1^{obsd}$) is given by

$$R_1^{obsd} = R_1^{HF} + R_1^{CSA} + R_1^{FeF} \qquad (7)$$

where R_1^{HF} represents the relaxation contribution of all H–F dipole–dipole interactions and the other terms correspond to the CSA and iron–fluorine effects. The $^{19}F\{^1H\}$ Overhauser effect for the system is

$$\eta = \frac{(\gamma_H/\gamma_F)\sigma_{HF}}{R_1^{HF} + R_1^{CSA} + R_1^{FeF}} \qquad (8)$$

where σ_{HF} represents cross-relaxation effects (Kalk & Berendsen, 1976) due to proton–fluorine dipolar interactions and the γ's are the appropriate gyromagnetic ratios. If we assume that the fluorophenylalanine rings are rigidly held within the globin structures and that the protein has a correlation time (τ_c) for isotropic tumbling of 30 ns, it can be shown that the numerator in eq 8 is equivalent to $-1.03R_1^{HF}$. Similarly, calculations based on these assumptions and the parameters provided by Hull & Sykes (1975) suggest that R_1^{CSA} is negligibly small relative to the experimental uncertainties in R_1^{obsd}. Thus, we can estimate R_1^{FeF} by

$$R_1^{FeF} \approx (1 + \eta/1.03)R_1^{obsd} \qquad (9)$$

Relaxation by a paramagnet is usually discussed in terms of the Solomon–Bloembergen equation (Dwek, 1973)

$$R_1^{FeF} = \frac{2}{15}\left(\frac{\gamma_F^2 g^2 S(S+1)}{r^6}\right)\left(\frac{3\tau_1}{1 + W_F^2\tau_1^2} + \frac{7\tau_1}{1 + W_{Fe}^2\tau_1^2}\right) \qquad (10)$$

Table III: Properties of Fluorine NMR Spectra

resonance	chemical[a,b] shift (ppm)	ionization shift (ppm)	pK_a	T_1[a,e] (s)	$T_2 \times 10^3$[a,f] (s)	$^{19}F\{H\}$ NOE	$\Delta W_{1/2}$ (Hz)[g,h] mod II	$\Delta W_{1/2}$ (Hz)[g,i] mod I
A1	15.63	0.13[d]	8.0[d]	0.41	2.9	−0.86	120	80
		−0.54	6.5					
A2	15.74	−1.61	7.40	0.41	3.0	−0.70	0	30
A3	15.17	−1.02	7.24	0.47	2.8	−0.53	0	20
A4	13.40	0.20[d]	8.0[d]	0.41	4.7	−0.80	0	0
		−0.55	6.0					
A5	~11.7[c]			0.43[c]		−0.7[c]	*c*	*c*
A6	10.16	−2.00	6.00	0.44	4.3	−0.76	0	0
A7	9.72	0.77	7.40	0.44	3.5	−0.60	0	>400
A8	7.12	0.33	7.40	0.09	2.5	−0.36	20	0
B1	19.02	0.11	7.30	0.05	1.4	−0.36	>400	>400
B2	18.36	−1.00	6.86	0.16	2.0	−0.55	200	200
B3	~11.7[c]			0.43		−0.7[c]	*c*	*c*
B4	~11.7[c]			0.43		−0.7[c]	*c*	*c*
B5	~11.7[c]			0.43		−0.7[c]	*c*	*c*
B6	~11.7[c]			0.43		−0.7[c]	*c*	*c*
B7	9.28	0.61	7.40	0.39	4.7	−0.50	40	75
B8	8.25	0.31	7.40	0.33	3.7	−0.53	0	50

[a] Sample was approximately 3 mM protein in 0.1 M Tris, 0.1 M NaCl, 0.01 M KCN, and 5% D_2O at pH 7.5. [b] Limiting chemical shift (complete ionization) relative to α-fluorocinnamic acid in methanol (capillary). [c] Overlapping resonances; the band evolves into several ill-resolved bands below pH 6.5, but the peak positions could not be reliably followed. [d] The first number given is for the step governed by K_1 and the second for the ionization defined by K_2. [e] Apparent spin-lattice relaxation time. [f] Estimated spin-spin relaxation time, from observed line widths at half-height. [g] Line-width changes produced by chemical modification of β-93 sulfhydryl groups. Samples were approximately 2 mM protein under the same conditions as described in footnote *a* of Table I. [h] Protein modified with compound II. [i] Protein modified with compound I.

Table IV: Data Derived from Fluorine NMR Spectra

resonance	chemical[a,b] shift (ppm)	temp dependence[d] × 10^2 (ppm/deg)	r_{FeF} (A)[e]	$r_{SL\text{-}F}$ (A)[f]
A1	−4.2	~0	8.6	>21*
A2	−4.2	~0	7.8	16
A3	−3.4	6.0	7.7	17
A4	−1.9	20	8.4	>21
A5	−0.1[c]	~0	−[c]	–
A6	1.4	~0	8.2	>21
A7	1.7	~0	7.8	<10
A8	4.4	−21	5.6	>21
B1	−7.3	11	5.0	>21*
B2	−6.8	23	6.4	>21*
B3	−0.1[c]	~0	−[c]	–
B4	−0.1[c]	~0	−[c]	–
B5	−0.1[c]	~0	−[c]	–
B6	−0.1[c]	~0	−[c]	–
B7	2.2	18.0	7.4	15*
B8	3.2	~0	7.8	14

[a] Sample described by footnote *a* of Table I. [b] Chemical shift relative to the signal from denatured protein in 6 M guanidine hydrochloride. A negative sign indicates a signal to lower field. [c] Overlapping resonances. [d] A negative temperature coefficient (δ/deg) indicates that the signal moves farther away (downfield) from the reference as the sample temperature increases. [e] Iron-fluorine distances computed as described in text. [f] Spin-label-fluorine distances estimated as described in text. The values marked with an asterisk correspond to signals that are appreciably altered by covalent modification of β-93 sulfhydryls with the diamagnetic material II. The distances >21 A are based on the assumption that a line-broadening effect 5 Hz or smaller could not be reliably detected.

where a term involving hyperfine interactions has been neglected. The correlation time τ_1 in this equation is composed of two terms:

$$\frac{1}{\tau_1} = \frac{1}{\tau_c} + \frac{1}{\tau_{Fe}} \qquad (11)$$

τ_c is the rotational correlation for tumbling of the protein, and τ_{Fe} is the relaxation time of the unpaired electron. Electron relaxation in cyanomethemoglobins is very rapid (Winter et al., 1972), and a reliable estimate of τ_{Fe} is not available. In order to gain some idea about the *relative* distance between the iron atom and the various fluorine nuclei, it was assumed that this distance for the closest interaction (resonance B1) is 5.0 Å, the smallest iron–fluorine distance computed for a variety of hemoglobin structures in the crystalline state, as discussed later. Assuming that all terms except the distance in eq 10 remain fixed from fluorine atom to fluorine atom we then have

$$r_{FeF}^{rel} = 5.0(16/R_1^{FeF})^{1/6} \qquad (12)$$

where r_{FeF}^{rel} is the relative iron–fluorine distance and the numerical factors in eq 11 are those for resonance B1. The relative internuclear distances calculated in this way are given in Table I; with the number of assumptions made these data probably provide a correct indication of the proximity of given fluorine atom to the heme iron, but the quantitative aspects should not be taken too seriously.

The transverse relaxation data (T_2) can be analyzed in a similar way although in this case the CSA contribution cannot be neglected and the $^{19}F\{^1H\}$ NOEs are not available to diagnose the fraction of relaxation arising from dipolar interactions. Those nuclei that are closest to the iron atoms, as indicated by the above analysis, also have the most efficient T_2 relaxation as would be expected if iron–fluorine interactions contribute to the process.

There are present a number of strongly contact-shifted resonances in the proton NMR spectrum of rabbit cyanomethemoglobin (Yamane et al., 1970). Signals centered around 15 ppm from tetramethylsilane and around 22 ppm are observed; the former have not been definitely assigned while the latter may arise from methyl groups attached to the heme (Morrow & Gurd, 1975). These two groups of proton signals were irradiated sequentially by a single proton frequency at a sufficient level (γ-$H_2/2\pi$ = 66 Hz) to saturate the resonances in one band but not perturb those in the other

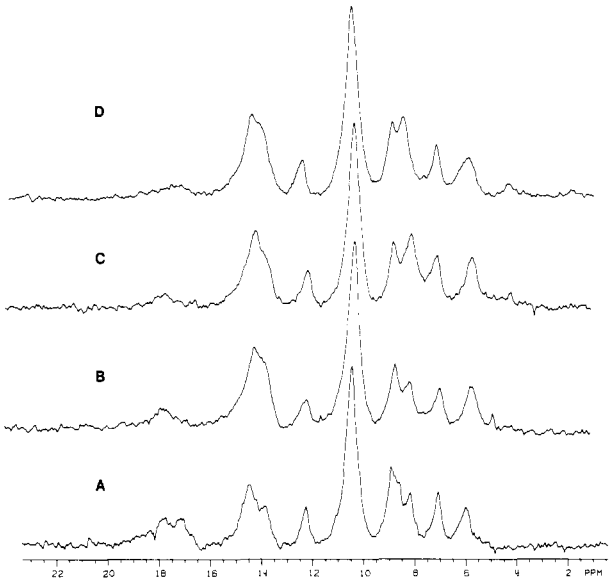
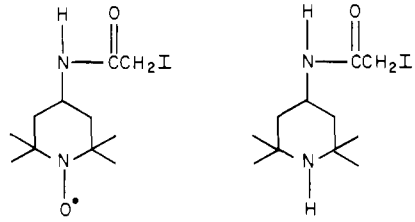


FIGURE 5: Effects of β-93 sulfhydryl modification on the fluorine NMR spectrum of fluorophenylalanine-containing cyanomethemoglobin. All spectra were obtained at pH 7.5 with solutions containing 0.1 M Tris, 0.1 M NaCl, 0.01 M KCN, and 5% deuterium oxide. (A) Native hemoglobin; (B) hemoglobin spin-labeled with I; (C) same as (B) but with the spin-label reduced with dithionite; (D) hemoglobin labeled with compound II.

or the bulk of the proton resonances farther upfield. Selective irradiation centered at 22 ppm had no effect on the intensities of the fluorine signals. However, irradiation of the proton signals near 15 ppm caused the group of resonances at 11.7 ppm (A4, B3–B6) to decrease in intensity an amount equivalent to a single fluorine resonance suffering a $^{19}F\{^1H\}$ NOE of about −0.7. These proton signals arise from nuclei attached directly to the heme ring or from amino acid protons structurally very close to the heme ring; the implication of this selective NOE is that at least one of the residues giving rise to the 11.7 ppm composite fluorine peak must lie near these shifted protons.

Modification of β-93 Sulfhydryl Groups. Compound I has



I II

been used in a number of spin-labeling studies of human hemoglobin (McConnell, 1971; Deal et al., 1971). The nitroxide function is expected to relax those atomic nuclei it comes close to in the protein structure (Jardetsky & Roberts, 1981), and we sought to use this approach to define the relative distances between the spin-label and the various fluorine nuclei of the rabbit fluorinated hemoglobin. As a control for these experiments protein labeled with II was prepared. Sulfhydryl group titrations indicate that either compound reacts with the two accessible sulfhydryl groups of the cyanomethemoglobin; these presumably are the two β-93 groups of the tetramer. The data in Figure 5 (trace D) show that modification with the diamagnetic analogue II has a pronounced effect on the fluorine spectrum of the hemoglobin. Shifts in lines A1–A3, A8, B7, and B8 are observed, and there are some line width changes, the most dramatic being the broadening of lines B1 and B2 to such an extent that these signals are nearly lost from the spectrum. Intensity corresponding to about 0.5 residue remains at ~18 ppm even after repeated exposure to the modification conditions, and it seems unlikely that this resonance represents unmodified protein.

Modification of the fluorinated hemoglobin with I produces the fluorine spectrum in trace B of Figure 5. Additional peak broadening due to the paramagnet is produced on several resonances, and signal A7 appears to be broadened so much that it disappears from the spectrum. Upon reduction of the spin-label A7 reappears in the spectrum coincident with B7 (trace C, Figure 5).

Spectra of the β-93-modified hemoglobins were analyzed by summing appropriate Lorentzian functions, and these analyses produced estimates of the fluorine line-width changes produced by these modifications. These are collected in Table IV and have estimated uncertainties of ±30%.

The apparent distance from the nitroxide function of protein modified with I to the various fluorine atom in fluorinated rabbit methemoglobin can be estimated by eq 10. [For an example see Arseniev et al. (1981).] The electron relaxation time for the nitroxide function was estimated to be 2 ns by using the relative intensities of the upfield and downfield hyperfine components of the ESR spectrum (Figure 1) of the spin-labeled hemoglobin (Kuznetsov et al., 1971). This value is in reasonable agreement with $\tau_e = 1.3$ ns found for spin-label I attached to human hemoglobin (Ohnishi et al., 1968). Since the rotational correlation time of the tetramer is expected to be about 30 ns, it is clear that the spin-label has significant mobility beyond that of simple tumbling of the protein. Spin-label-induced line broadening can be estimated by comparing the line-width effects of modification with I and II; the data in Table III permit the crude distance estimates in Table IV. These data, again, probably have only semiquantitative significance since the spin-label is not rigidly emplaced in the protein structure.

It should be emphasized that simply modifying the β-93 sulfhydryl groups is sufficient to move fluorine nuclei already proximate to heme iron even closer. In particular, the iron–fluorine distances for resonances B1 and B2 must be reduced by about a factor of 2.5 when the sulfhydryl groups are treated with I or II; smaller distance changes are found with resonances A1, A8, and B7.

Mn^{2+} Broadening. The fluorine NMR spectrum of the fluorinated rabbit hemoglobin was examined at pH 7.5 in the presence of increasing concentrations of manganous ion. Samples with up to 0.1 M Mn^{2+} were used; only a generalized broadening of all fluorine signals was observed with increasing Mn^{2+} concentration.

Discussion

The amino acid sequences for a number of vertebrate hemoglobins are available (Dayhoff, 1976). A striking aspect of these sequences is the very high degree to which the phenylalanine positions are conserved; in about 20 hemoglobins the positions α-33, α-36, α-43, α-46, α-98, α-117, α-128, β-41, β-42, β-45, β-45, β-71, β-85, β-103, β-118, and β-122 are nearly always occupied by phenylalanine. Of the mammalian hemoglobins, the protein from rabbit is unusual in that the α-48 position is polymorphic, usually being a phenylalanine but sometimes a leucine (Flamm et al., 1971; Garrick et al., 1974). X-ray crystallographic studies are available for a number of different forms of human and horse hemoglobin (Perutz, 1980; Baldwin & Chothia, 1979). Although the gross tertiary structures of these proteins in the crystal are similar, they are different in significant ways, and these differences

have been used in discussions, for example, of the structural correlates of ligation and the Bohr effect. Comparison of crystalline horse cyanomethemoglobin to the aquomet form shows that the ligand change has significant effects on local tertiary structure and, in particular, produces substantial reorientation of a phenylalanine residue near the heme groups in both globin chains (Deatherage et al., 1976).

Our evidence indicates that fluorophenylalanine in the diet substitutes randomly for phenylalanine at 16 positions; thus, all normally conserved phenylalanines in both globin chains and presumably Phe-α-48 are likely substituted. There is no evidence indicating tyrosine substitution and no evidence for strongly differential utilization for fluorophenylalanine by the two phenylalanine condon–anticondon systems used in rabbit hemoglobin synthesis (Heindall et al., 1978; Efstratiadis et al., 1977).

The question of possible substitution of D-*p*-fluorophenylalanine into proteins when a DL mixture is fed is of concern. We find that on a diet containing 0.15% of pure L isomer (the same amount present when the diet includes 0.3% of the DL mixture) the extent of incorporation is less than that observed with the DL mixture although the fluorine NMR spectra of the proteins isolated in these two cases are very similar. Given the great sensitivity of fluorine chemical shifts to structure we believe that it is unlikely that a protein containing both D- and L-*p*-fluorophenylalanine residues could have the same tertiary structure and, therefore, the same fluorine NMR spectrum as a protein containing only fluoro L-amino acid. There is no indication from spectra of bacterial proteins formed during growth on media containing DL mixtures of fluorinated amino acids that incorporation of both D and L stereoisomers takes place (Sykes & Weiner, 1980). Probably proteins formed in both the bacterial systems and in the rabbit have only L-fluorophenylalanine present. There is stereoselectivity in the absorption of amino acids in the gastrointestinal tract (Wilson, 1962), and D-fluorophenylalanine may be simply rejected at this stage. However, the higher levels of fluoro amino acid incorporation seen when the DL isomer is fed to the rabbit suggest that absorption, racemization, and, ultimately, utilization of the D form take place to some extent. This is consistent with the observation that D-phenylalanine in the diet is partially exploited for protein synthesis for rodents and man (Albanese, 1947). At the least, our experiments indicate that the D isomer of *p*-fluorophenylalanine is not any more toxic to the rabbit than the L form and possibly less so.

We have no choice but to interpret our fluorine NMR data in terms of the known tertiary structures. In doing so it will be assumed that rabbit hemoglobin in solution is not grossly dissimilar from the structure of crystalline horse and human hemoglobins. Given the sensitivity of the solid-state structures to relatively minor ligand changes we can expect only qualitative or perhaps semiquantitative guidance from these X-ray results.

Using published coordinates for horse aquomethemoglobin, human deoxyhemoglobin, and human (carbon monoxy)-hemoglobin (Protein Data Bank at the Brookhaven National Laboratory, the likely position of each *p*-fluorine atom was computed by appropriately extending the para C–H bond vector for each phenylalanine to give a C–F bond length of 1.3 Å. Distances between iron atoms and the fluorine nuclei were computed as were distances between the fluorines and the β-93 sulfur atom. For the Fe–F data reported in Table V, the distance to the closest heme iron was computed. Fluorine–sulfur distances given in Table III for fluorine in the α-globin subunit were for α^2–β^2 interactions; β^1–β^2 intersubunit distances were all greater than 20 Å. The pseudocontact shift at 25 °C for each fluorine was estimated by using the equations of Sheard et al. (1970). We note that, although these authors were dealing with myoglobin, the *g* factors used in their work were those observed with powdered samples of cyanomethemoglobin. The temperature dependence of each pseudocontact interaction was also computed, and these data are given in Table V. Finally, the influence of the heme ring current on chemical shifts was estimated by using the approximation developed by Shulman et al. (1970). The combined ring-current effect and pseudocontact contributions for each fluorine nucleus are given in Table V. Entries in Table V are averages of calculated data for the different crystalline hemoglobin structures mentioned; in most cases the range of a computed value was small, reflecting the similarity of these tertiary structures in the solid state. For the large pseudocontact shifts variations of a factor of 3 were noted in several instances (α-43, α-98, β-42, and β-103) due to the extreme sensitivity of the pseudocontact interaction to geometrical factors. In none of these cases was a reversal of sign noted for the computed shift effect.

Table V: Data Based on X-ray Coordinates[a]

residue	chemical shift (ppm)	temp dependence $\times 10^2$ (ppm/deg)	r_{FeF} (Å)	$r_{SL\text{-}F}$ (Å)
α-33	0.8	−1	8.1	16
α-36	0	0	14	16
α-43	7	−15	4.7	18
α-46	1	−3	8.4	22
α-48[b]	0.4	−1	11	19
α-98	−2	11	5.4	24
α-117	0	0	20	28
α-128	0.2	0	13	33
β-41	−0.4	2	10	11
β-42	5.0	−9	5	15
β-45	0.7	−1	8.5	20
β-71	0.7	−2	11	21
β-85	0.6	−2	10	16
β-103	−3	13	6.1	7.5
β-118	0	0	21	32
β-122	0	0	20	27

[a] Calculations were carried out for four structures as described in the text; values given are averages of those computed for these structures. [b] In human hemoglobin this residue is a leucine. A phenylalanine ring was placed in the structure with the aromatic side chain oriented in the same way as the alkyl side chain of leucine.

Comparison of the data in Tables IV and V permits us to make tentative assignments of many of the signals which appear in the fluorine NMR spectrum of *p*-fluorophenylalanine-containing rabbit hemoglobin. Considering the β-globin resonances first, it seems clear that resonance B1 arises from fluorophenylalanine β-103; a large downfield shift due to the pseudocontact term is predicted, the predicted sign and magnitude of the temperature coefficient of the chemical shift are correct, and the iron–fluorine distance is reasonably close to the calculated value. We cannot detect whether or not this resonance is appreciably relaxed by the nitroxide attached to the β-93 sulfhydryl, since it is strongly perturbed by chemical modification of this group alone. The computed close proximity of the sulfhydryl group to β-103 is consonant with this observation since any chemical modification at the sulfur could push the fluorine nucleus closer to the heme iron atom.

Resonance B2 is assigned to position β-41. Accepting the previous assignment leaves only one other residue with a computed downfield shift, a positive temperature coefficient

of the chemical shift, and reasonable proximity to both the iron and sulfur atoms. Clearly, the quantitative agreement is not as good as in the B1–β-103 combination, but it is close enough, when all four criteria are compared, to make this a convincing assignment as well.

The resonance for fluorophenylalanine β-42 is predicted to have a large upfield shift due to the heme ring current (~1.7 ppm) but also an appreciable pseudocontact interaction which has a negative temperature coefficient. It appears that resonance B7 may be due to this residue. A displacement of 2.4 Å away from the iron could reduce the pseudocontact effect, leaving only a modified (and reduced) upfield ring-current effect. Resonance B7 is affected by chemical modification of the β-93 sulfhydryl by both I and II, and some additional broadening by the spin-label is apparent. The smallest fluorine–sulfur distance remained after those of β-103 and β-41 is that of β-42, and it is primarily on these grounds that resonance B7 is assigned to β-42.

Spin-labeled human hemoglobin has been extensively studied, and X-ray studies of this protein modified with I have been reported (Likhtenshtein, 1976). In the solid the spin-label is found at the α–β interface where it is seen to occupy two positions, one with the nitroxide tip of the spin-label pointing toward the interior of the tetramer, moderately close to phenylalanine β-103, and the other with the nitroxide pointed away from the protein surface toward solution. Although the nitroxide function is doubtless mobile in solution, it seems likely that its conformational itinerary would include these positions and fluorine nuclei close to the α–β interface will be more strongly relaxed than those more buried in the protein. With this assumption the resonance B8 is assigned to fluorophenylalanine β-85; consideration of a model of hemoglobin indicates that, after β-103, this residue lies closest to the probable average position of the nitroxide group. A small upfield chemical shift is predicted for this fluorine due to the heme ring current and the pseudocontact interaction. A larger upfield effect than predicted is observed, and this may arise from aromatic ring currents generated by the β-71 and β-103 residues which are close.

Four β-globin resonances (B3–B6) are degenerate or nearly so under all of the experimental conditions examined so far. The chemical shifts for these are close to that observed for *p*-fluorophenylalanine residues exposed to solvent. Of the remaining unassigned residues, β-45, β-118, and β-122 lie close to the surface of the protein. Fluorophenylalanine β-45 is expected to be close to the heme ring (Perutz et al., 1968), and this may be the resonance in the B3–B6 set which experiences the $^{19}F\{^1H\}$ Overhauser effect when the contact-shifted heme proton signals are irradiated.

Although the averaged data recorded for residue β-71 suggest that a fluorophenylalanine at this position should be influenced by the heme ring and perhaps the spin-label attached to β-93, the location of this residue in the X-ray structures used to compute the data in Table V was the most variable of any in the β-globin chain. Thus, the computed iron–fluorine distance ranged from 10 to 14 Å and the calculated sulfur–fluorine distance from 14 to 31 Å. It is, therefore, easy to accept the notion that β-71 is able to take up an orientation in rabbit cyanomethemoglobin that has no distinguishing aspects that can be probed by our experiments and that the fluorine signal from this position appears in the B3–B6 set.

Resonance A8 experiences a large upfield shift and a large, negative temperature dependence and arises from a fluorine nucleus close to the iron atom in the α-globin. The data in Table V suggest that this signal is due to fluorophenylalanine at position α-43. There is no detectable effect of the spin-label on this resonance.

Table VI: Summary of Tentative Assignments

resonance	residue	resonance	residue
A1	α-33	B1	β-103
A2	α-46 or α-48	B2	β-41
A3	α-48 or α-46	B3	β-45
A4	α-98	B4	β-71
A5	α-117	B5	β-118
A6	α-128	B6	β-122
A7	α-36	B7	β-42
A8	α-43	B8	β-85

The resonance corresponding to fluorophenylalanine at α-98 is expected to be a pseudocontact shifted to low field, have a positive temperature coefficient, and be far enough from β-93 that a spin-label attached there has no effect. Resonance A4 meets these specifications and is thus assigned to α-98. However, resonance A3 has properties that agree nearly as well except that a small broadening effect of the spin-label is noted, and if the label is located at the α–β interface, this is not consistent with the position of α-98 in the structure.

One α-globin resonance contacts the spin-label sufficiently to become broadened beyond detection (resonance A7). Consideration of a molecular model of hemoglobin shows that phenylalanines α-33 and α-36 are closest to the α–β interface in the region where the spin-label is expected to reside. Iron-induced relaxation effects suggest that α-33 is the residue that is involved, but at this point we cannot choose confidently between these possibilities. However, we note that one α-globin residue is appreciably perturbed when the β-93 sulfhydryl is modified with the diamagnetic analogue II and suggest that this could be fluorophenylalanine α-33; the para position of this fluorophenyl ring is oriented toward the heme ring, and crowding introduced at the α–β interface by a tetramethylpiperidine ring attached to β-93 could easily shorten the iron–fluorine distance for this residue. We also note that the region of the structure containing the α-33 residue is unusually crowded, a situation which could result in the large, downfield shift this residue experiences through the van der Waals effect (Rummens, 1976). Thus, we have assigned resonance A1 to α-33 and thereby concluded that resonance A7 arises from α-36.

Fluorophenylalanines α-46 and α-48 are also reasonably close to the α–β interface and potentially influenced by a spin-label attached to β-93. The small line broadening produced by the spin-label indicates that resonances A2 and A3 should be assigned to α-46 and α-48, but there are no grounds for distinguishing the two possible assignments. The observed chemical shift effects to lower field are large, and there is no apparent reason for these; the local structure in this part of the α-globin must be quite different from that predicted from the solid-state structures.

Residues α-117 and α-128 are near the periphery of the α-globin molecule and should experience near normal chemical shifts. Both positions are removed from the expected spin-label position. α-128 is closer to the heme and should experience a small upfield shift due to the pseudocontact interaction. We therefore, assign resonance A6 to α-128, leaving A5 as the signal for α-117.

Table VI summarizes the assignments that have been made. We regard these as tentative, and further experimental work will be necessary to have them be equally convincing. However, we doubt that further evidence will change the assignments of A8, B1, or B2.

Some support for the assignments made comes from the pH dependencies observed. Major fluorine chemical shifts in this system arise from ring-current or pseudocontact interactions with the heme rings, and there are a number of histidines in the globins which undergo ionization in the pH range investigated. Should an ionization event appreciably alter a heme–fluorine distance, a large titration shift could be expected. Ohe & Kajita (1980) have determined the pKs for most histidine residues in human deoxy- and (carbon monoxy)hemoglobin, and with the exception of histidine α-89, the pKs are not strongly altered by ligation. The histidine residues discussed below are conserved in the rabbit globins, and we assume that the pKs of the human protein can be transferred to the rabbit system. Resonance A7, assigned to fluorophenylalanine α-36, titrates with a pK of 7.4 and a chemical shift change of 0.77 ppm upon ionization. This group is expected to be quite close to histidine-103; the latter residue has a pK of 7.1 in the human system. Fluorophenylalanines α-46 and α-48 are close to histidine α-50 which titrates with a pK of 7.2. Resonances A2 and A3 have been assigned to these residues and show large titration shifts with pK = 7.2 and 7.4. These resonances overlap at some pH values so that the pK values computed are likely indistinguishable within our errors. Fluorophenylalanines α-98 and α-117 are proximate to histidines α-87 and α-122, respectively. These histidines do not titrate in the human protein (Ohe & Kajita, 1980), and only small changes in the chemical shifts of these resonances, assigned to A4 and A5, are observed. Resonance A6 shows a large chemical shift effect upon ionization and an anomalously low pK. If our assignment of this residue to α-128 is correct, there are no histidines nearby, and the ionization behavior of this resonance is not interpretable at this point.

Fluorophenylalanine β-41 is near histidine α-45 and β-97. The former does not titrate in the human protein while the latter has a pK of 6.4. Resonance B2 assigned to β-41 shows a strong titration with pK = 6.9, and the pH behavior is thus consistent with our assignment. β-Globin residues β-42 and β-45 are close to histidine β-63, and fluorophenylalanine β-103 is close to histidine β-92. However, these histidines do not titrate in the human protein, and we note only small titration shifts on the fluorine signals assigned to these groups.

A valid concern when fluorine is substituted in biological systems is the extent to which the native structure is perturbed. In the present work the replacement of phenylalanine by fluorophenylalanine was kept low so that, on average, only one substitution per tetramer takes place. Our system thus essentially consists of a mixture of 16 isomeric proteins differing only in the position of the fluorinated amino acid. That some progress toward assigning the resonances can be made by considering the solid-state structures of the closely related human protein indicates that the tertiary structure of the rabbit protein is similar to the human and horse proteins, that the structure does not change drastically when placed in solution, and that the various fluorine substitutions do not greatly perturb the structure. The kinetics of ligation of rabbit hemoglobin suggest that the rabbit and human β-chains are very similar in their reactivities (Sharma et al., 1980). Greater differences are seen when the α-chains are compared, and it is with the α-chain where we have more difficulty in making assignments.

Chemical modification of the β-93 sulfhydryl group of horse oxyhemoglobin results in a number of perturbations of the tertiary structure in the crystalline state, with changes being observed in amino acid positions up to 25 Å from the β-93 residue (Moffat, 1971). NMR studies of the hyperfine-shifted resonances of human hemoglobin indicate that modification of β-93 induces localized conformational changes within the β-subunit (Neya & Morishima, 1980). Our observations that attachment of α-iodoacetamido compounds to β-93 produces large changes in the fluorine NMR spectrum of fluorinated rabbit hemoglobin are, thus, not unanticipated. The apparent alteration in the structure of the α-subunit produced by the reaction is, to our knowledge, without precedent and reminds us again that attachment of a spin-label or other probe to a protein cannot be assumed to be benign.

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Registry No. L-*p*-Fluorophenylalanine, 1132-68-9; L-phenylalanine, 63-91-2; fluorine-19, 14762-94-8.

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Characterization of the Complementary Deoxyribonucleic Acid and Gene Coding for Human Prothrombin†

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ABSTRACT: The DNA sequences of a complementary deoxyribonucleic acid (cDNA) and a portion of the gene coding for human prothrombin have been determined. The cDNA was 2005 base pairs in length and was found to code for part of a leader sequence of 36 amino acids, 579 amino acids present in the mature protein, a stop codon, a noncoding region of 97 base pairs, and a poly(A) tail of 27 base pairs. It is proposed that the leader sequence consists of a signal sequence and a pro sequence for the mature protein that circulates in plasma. The 10 glutamic acid residues that are present in the amino-terminal region of prothrombin and are converted to γ-carboxyglutamic acid in the mature protein are coded by only the GAG codon. The cDNA for prothrombin was also employed as a probe for screening a human fetal liver genomic DNA library. One of the strongly positive phage containing a human DNA insert of 5 kilobases was mapped with restriction endonucleases and sequenced. This DNA contained approximately half of the gene for human prothrombin and included six introns and five exons coding for amino acid residues 144–448. The two largest intervening sequences in the genomic DNA contained two copies each of *Alu*I repetitive DNA.

Prothrombin (M_r 72 000) is a vitamin K dependent protein that participates in the final phase of blood coagulation (Mann & Elion, 1980). It is synthesized in the liver and secreted into the blood where it participates in the coagulation process. When coagulation is initiated, prothrombin is converted to thrombin by minor proteolysis by factor X_a (activated Stuart factor) in the presence of factor V_a (activated proaccelerin), calcium ions, and phospholipid. Thrombin then converts fibrinogen to an insoluble fibrin clot. The amino acid sequences for bovine and human prothrombin have been reported (Magnusson et al., 1975; Butkowski et al., 1977; Thompson et al., 1977; Walz et al., 1977; Seegers, 1979). Each protein contains approximately 8% carbohydrate, including three carbohydrate chains and 10 residues of γ-carboxyglutamic acid

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