990-995.
Dayhoff, M. E., Ed. (1972), Atlas of Protein Sequence and Structure, Vol. 5, Silver Springs, Md., National Biomedical Research Foundation.
Daughaday, W. H., Hall, K., Raben, M. S., Salmon, W. D., Jr., Van den Brande, J. L., and Van Wyk, J. J. (1972), *Nature (London) 235*, 107-109.
Daughaday, W. H., Phillips, L. S., and Herrington, A. C. (1975), *Methods Enzymol. 37B*, 93-96.
Dias da Silva, W., and Lepow, I. H. (1967), *J. Exp. Med. 125*, 921-926.
Dias da Silva, W., Eisele, J. W., and Lepow, I. H. (1967), *J. Exp. Med. 126*, 1027-1048.
Edelhoch, H. (1967), *Biochemistry 6*, 1948-1954.
Edman, P. (1960), *Ann. N.Y. Acad. Sci. 88*, 602-610.
Ellmann, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70-77.
Fernandez, H. N., and Hugli, T. E. (1976), *J. Immunol. 117*, 1688-1694.
Hugli, T. E. (1975), *J. Biol. Chem. 250*, 8293-8301.
Hugli, T. E., and Erickson, B. W. (1976), *Proc. Natl. Acad. Sci. U.S.A. 74*, 1826-1830.
Hugli, T. E., and Müller-Eberhard, H. J. (1978), *Adv. Immunol. 26* (in press).
Hugli, T. E., Morgan, W. T., and Müller-Eberhard, H. J. (1975a), *J. Biol. Chem. 250*, 1479-1483.
Hugli, T. E., Vallota, E. H., and Müller-Eberhard, H. J. (1975b), *J. Biol. Chem. 250*, 1472-1478.
Jacobs, J. W., and Niall, H. D. (1975), *J. Biol. Chem. 250*, 3629-3636.
Johnson, A. R., Hugli, T. E., and Müller-Eberhard, H. J. (1975), *Immunology 28*, 1067-1080.
Laemmli, V. K. (1970), *Nature (London) 227*, 680-682.
Marshall, R. N., Underwood, L. E., Voina, S. J., Foushee, D. B., and Van Wyk, J. J. (1974), *J. Clin. Endocrinol. Metab. 39*, 283-291.
Pisano, J. J., and Bronzert, T. J. (1969), *J. Biol. Chem. 244*, 5597-5610.
Salmon, W. D., and Daughaday, W. H. (1957), *J. Lab. Clin. Med. 49*, 825-831.
Sauer, R. T., Niall, H. D., Hogan, M. L., Keutmann, H. T., O'Riordan, J. L. H., and Potts, J. T., Jr. (1974), *Biochemistry 13*, 1994-1999.
Van Wyk, J. J., Underwood, L. E., Hintz, R. L., Viona, S. J., and Weaver, R. P. (1974), *Recent Prog. Horm. Res. 30*, 259-295.
Wuepper, K. D., Bokisch, V. A., Müller-Eberhard, H. J., and Stoughton, R. B. (1972), *Clin. Exp. Immunol. 11*, 13-20.

Tyrosyl–Base–Phenylalanyl Intercalation in Gene 5 Protein–DNA Complexes: Proton Nuclear Magnetic Resonance of Selectively Deuterated Gene 5 Protein†

Joseph E. Coleman* and Ian M. Armitage

ABSTRACT: The interactions of oligodeoxynucleotides with the aromatic residues of gene 5 protein in complexes with $d(pA)_8$ and $d(pT)_8$ have been determined by ^1H NMR of the protein in which the five tyrosyl residues have been selectively deuterated either in the 2,6 or the 3,5 positions. Only the 3,5 protons of the three surface tyrosyls (26, 41, and 56) interact with the bases. The remainder of the aromatic protons undergoing base-dependent upfield ring-current shifts on complex formation are phenylalanyl protons, assigned to Phe(13) on the basis of model building. ^{19}F NMR of the complexes of the *m*-fluorotyrosyl-labeled protein with $d(pT)_4$ and $d(pA)_8$ confirms the presence of ring-current perturbations of nuclei at the 3,5-tyrosyl positions of the three surface tyrosyls. Differential expression of the $^{19}F\{^1H\}$ nuclear Overhauser effect confirms the presence of two buried and three surface tyrosyl residues. A new model of the DNA binding groove is presented involving Tyr(26)-base-Phe(13) intercalation.

Gene 5 protein is a single-stranded DNA-binding protein elaborated by *Escherichia coli* infected with fd bacteriophage and coded for by the phage genome (Alberts and Frey, 1970; Alberts et al., 1972; Oey and Knippers, 1972). Mutants of fd temperature sensitive in the gene 5 product show that gene 5 protein is required for the shift from double-stranded DNA synthesis of the replicative forms (RF) to single-stranded DNA synthesis of the daughter viral genomes (Salstrom and Pratt, 1971; Mazur and Model, 1973). Gene 5 protein binds to single-stranded DNA of any sequence, although binding studies with defined oligodeoxynucleotides show it to have a much greater affinity for adenine-rich regions than for thymine-rich regions (Coleman et al., 1976). A variety of titration studies using spectroscopic detection of complex formation have shown that each gene 5 monomer (M_r = 9689) interacts with ca. four bases (Alberts et al., 1972; Pratt et al., 1974; Anderson et al., 1975). In the presence of low salt, gene 5 protein will melt certain double-stranded homopolymers at room temperature, e.g., poly[d(A-T)], since the equilibrium is far in favor of the gene 5 protein complex with the single strand. On interaction with circular single-stranded fd DNA, both in vivo and in vitro

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gene 5 protein collapses the circular single strand into a cigar-shaped structure containing two antiparallel strands of DNA (Pratt et al., 1974).

Since detailed spectroscopic, chemical-modification, and NMR studies show that a tetranucleotide mimics almost all specific interactions of a polynucleotide, the likelihood is that the two antiparallel DNA strands in the complex are held together by protein–protein interactions. A model such as that pictured in Figure 1, in which a gene 5 protein dimer might be considered like a primitive histone complex on which the strands are wrapped, would account for the present findings. Additional supercoiling, which may give rise to the ~160-Å diameter of the in vivo complex as observed by electron microscopy, was not attempted in this simple model in which the DNA binding grooves are arbitrarily placed at the maximum distance across the twofold axis of the dimer.

The interactions of the DNA single strand along the binding site on the protein have been the subject of extensive ^{1}H NMR studies showing that a large number of the aromatic protons undergo chemical shifts on complex formation (Coleman et al., 1976). Gene 5 protein contains five Tyr, three Phe, one His, and no Trp residues. Nitration of the protein results in nitration of three of the five tyrosyl residues in the protein, Tyr-26, -41, and -56. Nitration of these residues is completely prevented by binding of a tetranucleotide. In addition ^{19}F NMR studies of gene 5 protein in which the tyrosyl residues were labeled with *m*-fluorotyrosine identify three surface tyrosyls which interact with oligodeoxynucleotides (Anderson et al., 1975). These data, combined with the base-dependent upfield shifts observed for ~35% of the aromatic protons of gene 5 protein on complex formation, were used to propose a model in which the bases of the nucleotide were pictured as intercalating with tyrosyl-26, -41, and -56 (Coleman et al., 1976). The aromatic protons which shifted on complex formation were assigned to the 2,6 and 3,5 protons of the tyrosyl residues.

Precise assignments of individual resonances in the aromatic ^{1}H NMR spectrum of a protein are difficult, especially distinguishing resonances from the 2,6 protons of tyrosyl from those of phenylalanyl residues. Assignment can be made more precise by selectively deuterating individual groups of aromatic protons such as the 3,5- or the 2,6-tyrosyl protons. Spectra of gene 5 protein carrying selectively deuterated aromatic residues have required a reassignment of the aromatic resonances and indicate a more complex interaction of the aromatic residues with the nucleotides involving both tyrosyl and phenylalanyl residues. Combining the ^{1}H NMR data on the selectively deuterated nucleotide complexes with ^{19}F NMR data on complexes of the *m*-fluorotyrosyl-labeled protein with defined oligodeoxynucleotides allows a more detailed model of the interaction of the nucleotide with aromatic residues of the protein to be made than was previously possible. A preliminary account of the ^{19}F NMR of the fluorotyrosyl protein was presented to the British Biophysical Society Meeting, Oxford (Coleman and Armitage, 1977).

Materials and Methods

Gene 5 Protein. Homogeneous gene 5 protein was prepared by DNA–cellulose chromatography (Anderson et al., 1975). For calculations of the concentration of gene 5 protein, an $E_{276nm}^{0.1\%} = 0.73$ was employed (Day, 1973). pH refers to the direct reading on a radiometer pH meter (GK2023C electrode) taken on H_2O or D_2O solutions. Protein solutions in 99.8% D_2O were prepared by repeated lyophilization from dilute buffer made up in D_2O.

Deuterated tyrosine was prepared by the method of Griffiths et al. (1976), and gene 5 protein was isolated from a tyrosine auxotroph of an Hfr strain of *E. coli* infected with fd (Anderson et al., 1975) growing on a pure amino acid medium containing the deuterated tyrosine.

FIGURE 1: Proposed model of a section of a gene 5 protein–DNA complex in which the antiparallel strands of DNA are held together by protein–protein interactions between gene 5 monomers in the dimer.

Nucleotides. Tetra- and octanucleotides of defined sequences were purchased from Collaborative Research (Waltham, Mass.), dissolved in 99.8% D_2O, and extracted with a CCl_4 solution of dithizone to remove paramagnetic metals. Nucleotides were added to the protein solutions by lyophilizing the correct volume and concentration of nucleotide from D_2O buffer and adding the lyophilized nucleotide to the protein solution.

NMR Methods. ^{1}H NMR spectra were recorded on a Bruker HX270 spectrometer interfaced to a Nicolet BNC-12 computer and a Nicolet 293I/O controller. D_2O present in the sample served as a field-frequency lock. Irradiation of the proton resonance of water was carried out by the gated decoupling technique (Hoult and Richards, 1975). Measurements were made at 20 °C, and chemical-shift values are reported as parts per million (ppm) downfield from sodium 4,4-dimethyl-4-silapentanesulfonate. Samples were contained in 0.01 M DPO_4^{2-} (pH 8.0) at 25 °C.

^{19}F NMR spectra were recorded on a FT-Bruker HFX-90 spectrometer operating at 84.6 MHz. For the gated decoupled NOE spectra, a flip angle of 90° and a pulse delay of 0.3 s was employed. D_2O present in the sample served as the field-frequency lock, and chemical shifts are reported in parts per million relative to CF_3COOH. Solution conditions were 0.01 M Tris-HCl,[1] 90% H_2O–10% D_2O (pH 8.0) at 25 °C. The convention of negative chemical shifts to high field of the standard for all nuclei is used throughout.

Results

^{19}F NMR of Complexes of Gene 5 Protein with $d(pT)_4$ and $d(pA)_8$. Previous ^{19}F NMR studies of gene 5 protein biosynthesized with *m*-fluorotyrosine identified two groups of tyrosyl residues in the protein, two fluorotyrosyl residues with resonances downfield from that expected for free *m*-fluorotyrosine and three with resonances near that of free *m*-fluorotyrosine (Figure 2A). The former were assigned to tyrosyl residues 34 and 61, known from chemical-modification studies to be relatively buried in the protein structure, while the latter were assigned to tyrosyl residues 26, 41, and 56, postulated to be on the surface of the protein, since they can be nitrated and acetylated (Anderson et al., 1975). Resonances of the three "surface" residues move upfield by 0.1 to 0.3 ppm on formation of complexes between gene 5 protein and tetranucleotides of

[1] Abbreviation used: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

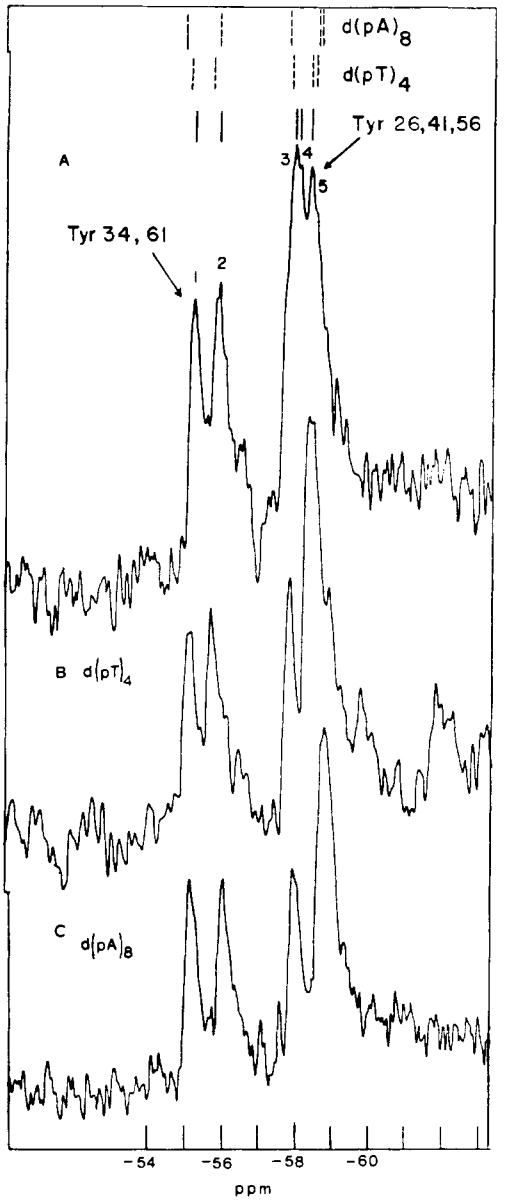

FIGURE 2: ^{19}F NMR of *m*-fluorotyrosyl gene 5 protein, 5×10^{-4} M (A), plus 1×10^{-3} M $d(pT)_4$ (B), plus 1×10^{-3} M $d(pA)_8$ (C).

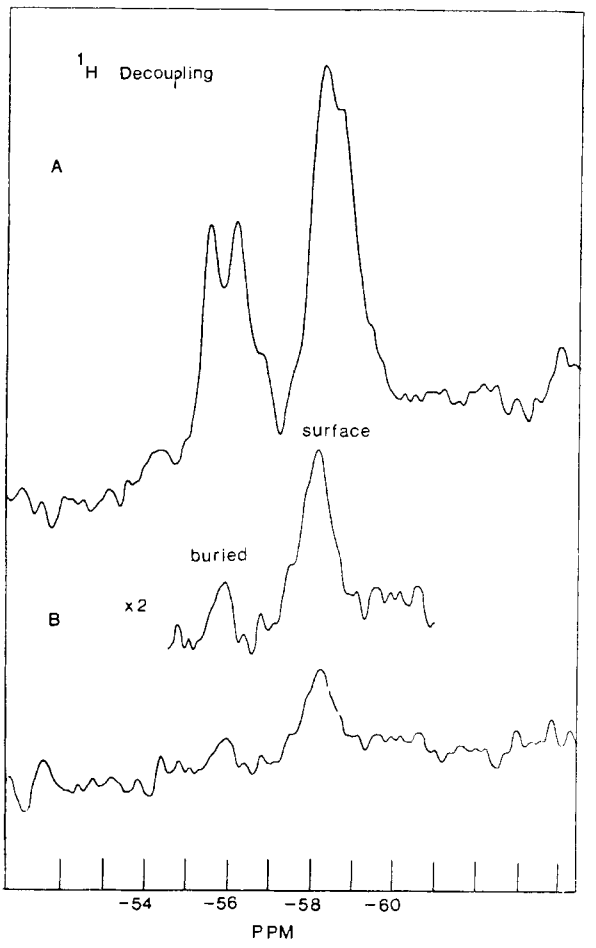

FIGURE 3: (A) ^{19}F NMR of *m*-fluorotyrosyl gene 5 protein, 5×10^{-4} M, under pulsing conditions for decoupling, without ^1H decoupler on, 10-mm sample tube. (B) Same with ^1H decoupling.

random sequence (Coleman et al., 1976). These chemical shifts were interpreted as base-induced ring-current shifts, evidence supporting the hypothesis that the surface tyrosyl residues are involved in stacking interactions with the bases of the tetranucleotide.

Use of tetra- and octanucleotides of defined sequence to form complexes with fluorotyrosyl gene 5 protein allows a more detailed examination of the changes in chemical shift of the resonances of the three surface tyrosyl residues on formation of the nucleotide complexes. ^{19}F NMR spectra of the gene 5 complexes with $d(pT)_4$ and $d(pA)_8$ are shown in Figures 2B and 2C and compared to the spectrum of the unliganded protein (Figure 2A). With oligonucleotides of defined sequence the upfield chemical shifts are larger and the resonances of the surface residues in the complex are more completely separated than with nucleotides of random sequence. It is clear that only two of these resonances participate in upfield shifts, while the third resonance remains relatively unshifted [$d(pT)_4$] or actually shifts downfield slightly [$d(pA)_8$]. The downfield resonances ("buried tyrosyls") also move slightly downfield, more so in the $d(pA)_8$ complex. The changes in chemical shift of the ^{19}F resonances on complex formation are tabulated in Table I.

$^{19}F\{^1H\}$ Nuclear Overhauser Effects (NOE) in Gene 5 Protein. In a detailed analysis of the relaxation behavior of fluorine nuclei attached to alkaline phosphatase, Hull and Sykes (1975) have demonstrated that dipolar interactions with protons dominate the spin-lattice relaxation mechanism. Thus, a measurement of the $^{19}F\{^1H\}$ NOE provides a convenient way to determine the contribution from the dipolar mechanism and, furthermore, provides an independent determination of the dynamics of motion of the fluorine-containing residue. The maximum $^{19}F\{^1H\}$ NOE in the presence of rapid isotropic molecular motion is +0.53 (Noggle and Schirmer, 1971). As discussed extensively by Hull and Sykes (1975), expression of the $^{19}F\{^1H\}$ NOE is dependent on the magnitude of the rotational correlation time modulating the H–F vector. As τ_c becomes longer, the NOE varies from a maximum of +0.53 to a minimum of −1.06. An NOE of −1 implies complete loss of the ^{19}F signal on proton irradiation. For immobilized tyrosyl residues contained in large protein molecules with rotational reorientation times of $\sim 10^{-8}$ s, the NOE assumes its minimum value and the ^{19}F resonances disappear on proton decoupling (Hull and Sykes, 1975). Even for a protein with τ_c greater than 10^{-8} s, however, the presence of rapid internal rotation about a fluorotyrosyl C_α–C_β bond ($\tau_i = 10^{-8}$ to 10^{-10} s) will attenuate the NOE, and a resonance will remain on proton decoupling (Hull and Sykes, 1975).

TABLE I: ^{19}F Chemical Shifts[a] in Fluorotyrosyl Gene 5 Protein and its Complexes with $d(pA)_8$ and $d(pT)_4$.

resonance	$d(pT)_4$ complex (ppm)	$\Delta\delta$	gene 5 protein (ppm)	$\Delta\delta$	$d(pA)_8$ complex (ppm)
1 (buried Tyr)	−55.66	+0.14	−55.80	+0.26	−55.54
2 (34, 61)	−56.29	+0.14	−56.43	−0.04	−56.47
					−58.37
3 (surface Tyr)	−58.48		−58.57		
4 (26, 41, 56)			−58.57	−0.72[b]	
5	−59.00	−0.43[b]	−58.97		−59.29
	−59.00				−59.29

[a] Chemical shifts are accurate to ±0.06 ppm. The solid arrows indicate the maximum upfield shifts shown by the fluorines on the surface tyrosyls, while the dotted arrows indicate small downfield shifts that occur on formation of the nucleotide complexes. Conditions: 0.01 M Tris, pH 8.0, 10% D_2O, 25 °C. [b] The most upfield ^{19}F resonance position in the complexes is base dependent and contains two resonances (4 and 5) of the same chemical shift. On the other hand, in the unliganded protein, two resonances (3 and 4) have the same chemical shift and are located at the most downfield position of this group. Hence, the shifts, $\Delta\delta$, of −0.43 and −0.72 ppm will apply to at least one and likely both resonances (those labeled 3 and 4 in the unliganded protein). The alternate assignment of resonance 5 in the unliganded protein as one of those moving upfield on complex formation means that it moves upfield 0.3 ppm in the presence of adenine and only 0.03 ppm in the presence of thymine, a tenfold difference that appears unlikely. Note that the numbers 1–5 refer only to the order of resonances from downfield to upfield.

If gene 5 protein is assumed to be spherical, the monomer will have a radius of ~14 Å and an overall rotational correlation time, τ_c (Stokes–Einstein), of $\sim 8.4 \times 10^{-9}$ s. If, as seems likely, however, the dimer is the minimum molecular species present in solution (Pretorius et al., 1975), the long axis may be >50 Å. The slowest rotational correlation time of the dimer will involve tumbling about an axis perpendicular to the long dimension, and this τ_c will be $\sim 7 \times 10^{-8}$ s. In the absence of internal motion of the tyrosyls, τ_c of the monomer predicts that less than 20% of the ^{19}F resonance should remain on proton decoupling, while resonances from immobilized tyrosyls in the dimer should completely disappear.

The ^{19}F spectra of fluorotyrosyl gene 5 protein in the absence and presence of ^1H decoupling are shown in Figure 3. On proton decoupling, all tyrosyl resonances are substantially reduced, with less diminution of the resonances are substantially reduced, with less diminution of the resonances at the upfield position (surface tyrosyl) (Figure 3B). Qualitatively, this suggests that the surface tyrosyls have a greater degree of freedom of motion than the buried tyrosyls (see Discussion).

^1H NMR of Gene 5 Protein–Deoxynucleotide Complexes Containing Selectively Deuterated Tyrosyl Residues. The complete ^1H NMR spectra of gene 5 protein and representative deoxynucleotide complexes have been presented previously (Coleman et al., 1976; Coleman and Armitage, 1977). The present data focus on a precise assignment of the resonances arising from the protons of the aromatic amino acid residues by the technique of selective deuteration. Hence, only spectra of the aromatic protons are shown.

2,6 Deuteration. The ^1H NMR spectrum of gene 5 protein synthesized from 2,6-deuterated tyrosine is shown in Figure 4A and compared to that of the completely protonated protein. The difference between the two spectra identify two important regions of the spectrum. The 2,6 protons of the five tyrosyl residues occur between 6.7 and 7.3 ppm, a normal chemical shift for the 2,6 protons of tyrosine (cross-hatched region, Figure 4A). The effective elimination of the coupling between the 2,6 and 3,5 protons accompanying 2,6 deuteration results in a much better resolution of the 3,5 protons, peaks 8 and 9. Peak 9 at ~6.5 ppm is unusually far upfield; however, the observed narrowing and an integrated intensity corresponding to two protons confirms the assignment of this resonance to the 3,5 protons of a tyrosyl subject to ring-current shifts in the unliganded molecule (see Discussion).

For comparison, difference spectra summarizing the changes in the aromatic proton NMR spectra of the totally protonated protein on formation of complexes with $d(pT)_8$ and $d(pA)_8$ are shown in Figure 5. These have been presented in detail previously (Coleman et al., 1976). Resonances marked 3, 4, 7′, and 9 are those disappearing from the protein on complex formation. Resonance below the line indicates new resonance appearing in the complex. Resonance from peaks 3 (7.42 ppm) and 4 (7.35 ppm) in the unliganded protein appear to give rise to resonance ~0.3-ppm upfield in the complex with $d(pT)_8$ (peak 6 at 7.05 ppm) and 0.7- and 0.8-ppm upfield in the complex with $d(pA)_8$ (peak 11 at 6.8 ppm). These shifts can be compared to shifts observed on complex formation with the selectively deuterated protein. Complex formation with $d(pT)_8$ is employed throughout, since there are few proton resonances in the aromatic region of the ^1H NMR of this nucleotide which overlap with the aromatic spectrum of the protein. The binding of all deoxynucleotides results in similar changes in the aromatic region of the ^1H spectrum of gene 5 protein. Differences occur only in the magnitude of the upfield shifts, as illustrated in Figure 5 (Coleman et al., 1976).

The ^1H NMR spectra of the 2,6-deuterated protein and its complex with $d(pT)_8$ are overlaid in Figure 4B. The cross lined areas show the resonances in the protein which undergo chemical shifts. Resonances 8, 9, and part of 7 (7′) disappear. These must represent 3,5 protons of tyrosyl residues, probably on three different residues. They appear to shift upfield and broaden into peak 10 of the complex.

Since neither of the two spectra in Figure 4B contain any resonances from 2,6-tyrosyl protons, peaks 3 and 4, which shift upfield into the region of peak 6 on complex formation, pre-

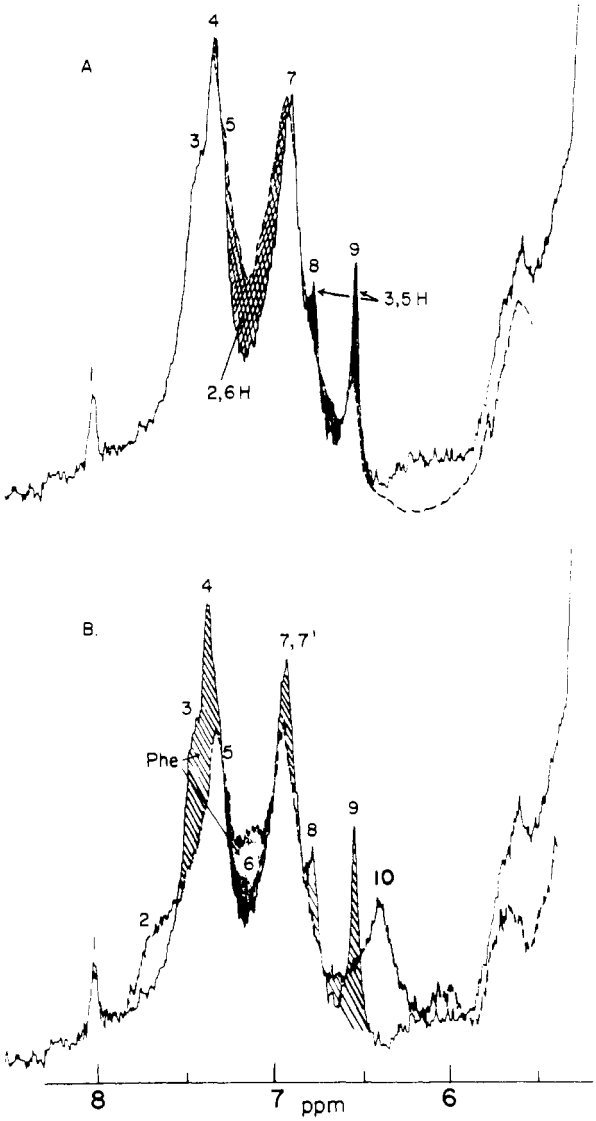

FIGURE 4: (A) (- - -) Aromatic ^{1}H NMR spectrum of totally protonated gene 5 protein, (—) spectrum of 2,6-deuterated tyrosyl protein, (▩) resonance assigned to 2,6-tyrosyl protons, (■) resonance assigned to 3,5-tyrosyl protons. (B) (—) 2,6-Deuterated tyrosyl protein, (- - -) plus 1 equiv of $d(pT)_8$, (▨) resonance disappearing from spectrum on complex formation, (■) new resonance appearing in the complex. All spectra here and in Figure 6 have been matched using peak 1, the resonance of the C(2)H of His-64, which is not affected by complex formation. All samples were 10^{-3} M protein, pH 8, 20 °C.

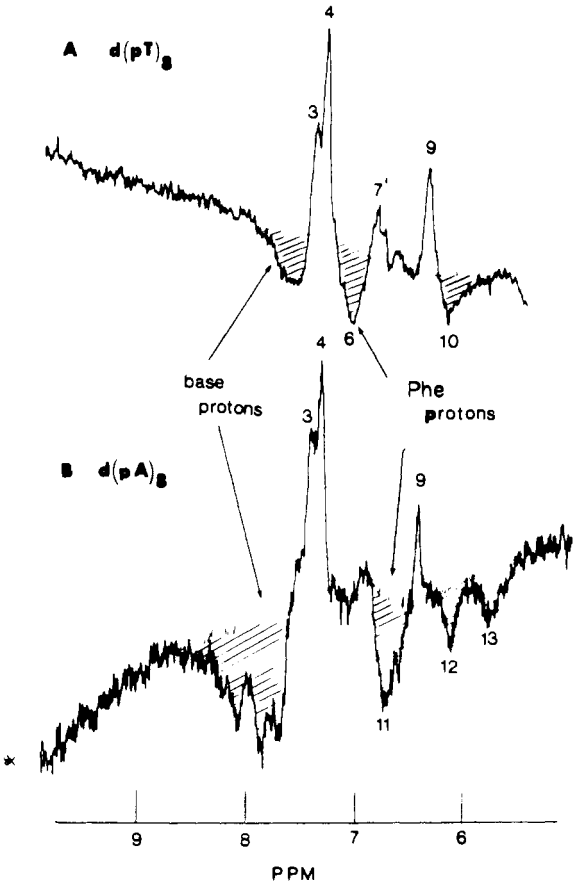

FIGURE 5: ^{1}H NMR difference spectra: (A) totally protonated gene 5 protein-$d(pT)_8$ complex, (B) totally protonated gene 5 protein-$d(pA)_8$ complex, (▭) resonance disappearing from the spectrum on complex formation; (▨) new resonance appearing in the complex.

sumably represent phenylalanine protons. Chemical shifts of 7.35 to 7.42 would be too far downfield for 3,5-tyrosyl protons. Already from a comparison of the spectra in Figure 4B with the previous difference spectra between the totally protonated protein and its complex (Figure 5), it is obvious that most of the chemical shifts of the aromatic protons are still present in the 2,6-deuterated tyrosyl sample. Thus, the protons most directly involved in nucleotide complex formation must be confined to the 3,5 protons of tyrosyl residues and phenylalanine protons. This assignment is confirmed by the spectra of the 3,5-deuterated tyrosyl derivative shown below.

3,5 Deuteration. The ^{1}H NMR spectrum of gene 5 protein biosynthesized on 3,5-deuterated tyrosine is shown in Figure 6A and compared to that of the completely protonated protein. Peaks 7, 9, and the region corresponding to peak 8 (crosslined in Figure 6A) have disappeared from the spectrum of the 3,5-deuterated protein, confirming the assignment of these resonances to 3,5 protons of the tyrosyl residues, as indicated by the spectra of the 2,6-deuterated derivative above (Figures 4A and 4B). Most of the resonance in peaks 3–5 remains in the 3,5-deuterated derivative. There is an alteration in the upfield envelope of these peaks (dark shaded area, Figure 6A), which probably represents a shift in the 2,6-tyrosyl proton envelope due to removal of the coupling to the 3,5 protons. Part of the resonance from the 2,6-tyrosyl protons occupies this region of the spectrum (Figure 4A). From the data in Figures 4 and 6 it appears likely that all of the 3,5 protons can be accounted for in peaks 7–9, which together account for 25–28% of the aromatic protons. Thus, additional resonances from tyrosyl 3,5 protons do not seem likely to occur in the upfield region between 7 and 8 ppm as originally thought.

Formation of the $d(pT)_8$ complex with the 3,5-deuterated protein results only in the shift of resonance from peaks 3 and 4 into the position of peak 6 (dark shaded area, Figure 5B). Thus, the majority of the resonance making up peaks 3 and 4 does not come from 2,6-tyrosyl protons (Figure 4A) or from 3,5-tyrosyl protons (Figure 6A) and must represent phenylalanyl protons which shift upfield into peak 6 on complex formation with $d(pT)_8$.

Difference Spectra of the Deuterated Proteins and their Respective $d(pT)_8$ Complexes. The quantitative aspects of the qualitative assignment of resonances 3 and 4 to Phe protons were confirmed by taking the difference spectra between the two deuterated gene 5 proteins and their corresponding $d(pT)_8$ complexes (Figure 7). These difference spectra were determined by setting the amplitude of the resonances of the C(2)H of His-64 the same in each set of spectra. All previous NMR

data show that this resonance is unaffected by complex formation (Coleman et al., 1976; Coleman and Armitage, 1977). Both difference spectra show the presence of resonances 3 and 4 in the protein (resonance above the base line) and their replacement by peak 6 in the complex (resonance below the base line). The presence of the 2,6 protons in the spectra derived from the 3,5-deuterated samples (Figure 7A) and the presence of the 3,5 protons in that derived from the 2,6-deuterated samples (Figure 7B) are indicated on the figures. Resonances 3 and 4 belong to neither group; hence, they must arise from the aromatic protons of phenylalanine.

Discussion

Chemical modifications of gene 5 protein which interfere with deoxynucleotide binding or which are prevented by prior binding of nucleotides to the protein have previously identified lysyl, tyrosyl, and cysteinyl side chains as located near the nucleotide binding site or directly interacting with the nucleotide (Anderson et al., 1975). Acetylation of the six lysyl ϵ-NH_2 groups of the protein prevents nucleotide binding. Acetylation of these residues is not blocked by DNA complex formation. These findings and the ability of cations to dissociate the complex have suggested that the positively charged ϵ-NH_3^+ groups participate in nucleotide complex formation by neutralizing the negative charges of the phosphate backbone. NMR studies of the complex show no chemical shift or enhanced relaxation of the ϵ-CH_2 protons on complex formation (Coleman et al., 1976). This suggests that, while the positively charged lysyl residues form a neutralizing charge cloud on the outside of the complex, the individual lysyl side chains must retain considerable mobility. This postulate is also compatible with the failure of nucleotide binding to prevent acetylation of the lysyl ϵ-NH_2 groups.

Nitration of tyrosyl residues 26, 41, and 56 prevents nucleotide binding, but in contrast to the lysyl modification, nitration is completely prevented by prior binding of DNA or a tetranucleotide to the protein. ^{19}F NMR studies of the protein labeled with *m*-fluorotyrosyl residues show the five tyrosyls of the protein to exist as two buried residues and three surface residues (Anderson et al., 1975; Figure 2). Both ^{19}F and ^1H NMR studies of the tyrosyl resonances show the surface tyrosyls to interact with tetra- and octanucleotides. The small, base-dependent, upfield chemical shifts occurring for resonances from both nuclei suggested that these tyrosyls intercalate with the bases (Coleman et al., 1976).

Mercuration of Cys-33 prevents nucleotide binding. The Hg(II) binding is prevented by prior binding of DNA. Hence, Cys-33 must be located in or near the DNA binding groove (Anderson et al., 1975).

These findings have previously been synthesized into a model for the DNA binding surface of gene 5 protein (Coleman et al., 1976). The secondary structure of the protein was constructed on the basis of the primary sequence data following the predictions of the Chou-Fasman analysis (Chou and Fasman, 1974; Anderson et al., 1975). The interactions of the DNA with specific residues of the protein along a DNA binding groove were then inferred from the chemical modification and NMR data.

While model building of the general structure of the protein from solution data is highly speculative, the interactions between the bases and specific amino acid side chains of the protein in oligodeoxynucleotide complexes can be defined rather precisely by the various NMR methods available.

In the initial proton NMR studies of nucleotide complex formation, ~12 of the aromatic protons of gene 5 protein were

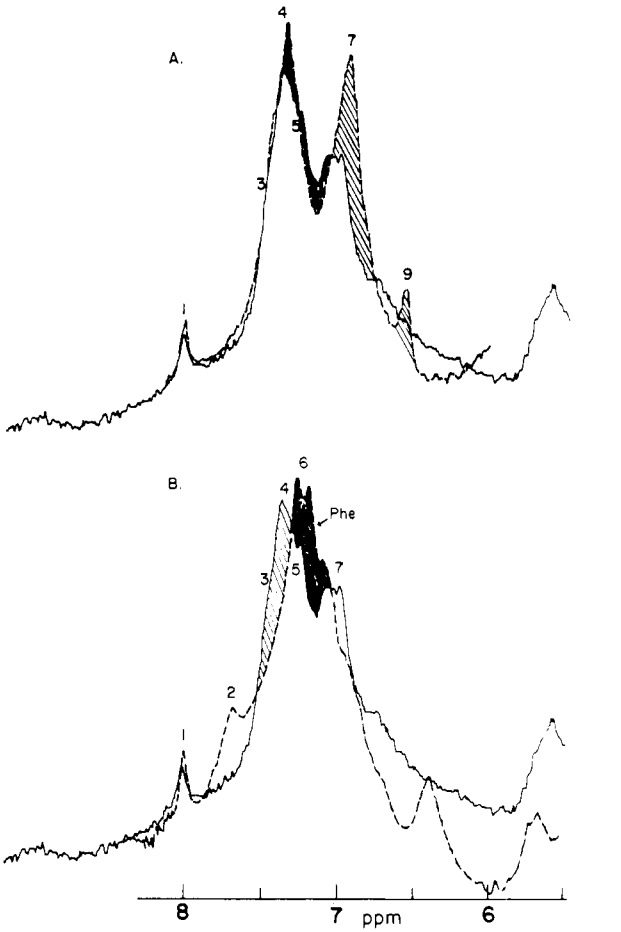

FIGURE 6: (A) (- - -) Aromatic ^1H NMR spectrum of totally protonated gene 5 protein, (—) spectrum of 3,5-deuterated tyrosyl protein, (▨) resonance assigned to 3,5-tyrosyl protons, (■) resonance disappearing from 3,5-deuterated protein and assigned to 2,6-tyrosyl protons undergoing a change in coupling from ^1H to ^2H. (B) (—) 3,5-Deuterated tyrosyl protein, (- - -) plus 1 equiv of $d(pT)_8$, (▨) resonance disappearing from the spectrum on complex formation, (■) new resonance appearing in the complex. Conditions as in Figure 4.

observed to move on nucleotide binding. In view of the chemical-modification studies implicating Tyr-26, -41, and -56 in nucleotide binding, these were assigned to the 2,6 and 3,5 protons of these three residues. The selective deuteration experiments show this assignment to be in error (Figures 4 and 6). Only the 3,5-tyrosyl protons are involved, and a major group of protons undergoing upfield shifts belong to at least one phenylalanyl residue (Figure 7). Examination of the previous model showed that Phe-13 was located very near Tyr-26 and could easily be rotated into the DNA-binding surface. The model of this surface has thus been altered to include Phe-13 in the alternating base-Tyr-base-Phe stack and reduces the extent to which the tyrosyls intercalate such that only the 3,5-protons come into van der Waals contact directly over the base rings (Figure 8). In the absence of the nucleotide, the aromatic ring of Phe-13 could stack over that of Tyr-26. This would account for the unusual upfield shift of one pair of 3,5 protons in the unliganded protein (Figure 4A). Only the 3,5 protons appear involved, which is the reason for the placement of Phe-13 relative to Tyr-26 (Figure 8). Loss of this interaction on nucleotide binding is consistent with the large loss in optical activity of the tyrosyl chromophores observed on nucleotide binding (Anderson et al., 1975). The close approach of Phe-13 to Tyr-26 could contribute to the induced ellipticity of the tyrosyl chromophores in the unliganded protein.

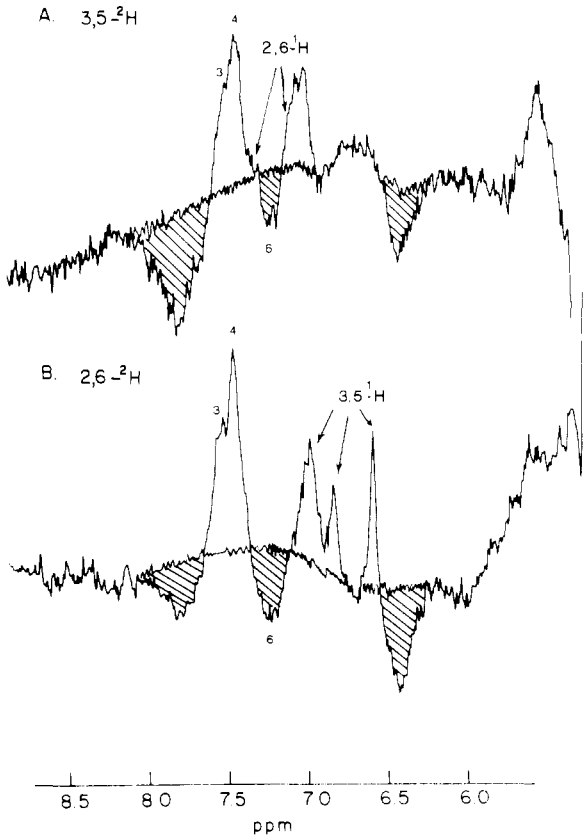

FIGURE 7: ^{1}H NMR difference spectra: (A) 3,5-deuterated tyrosyl gene 5 protein (3,5-deuterated tyrosyl gene 5 protein-d(pT)$_8$ complex); (B) 2,6-deuterated tyrosyl gene 5 protein (2,6-deuterated tyrosyl gene 5 protein-d(pT)$_8$ complex), (▭) resonance disappearing from the spectrum on complex formation, (▨) new resonance appearing in the complex.

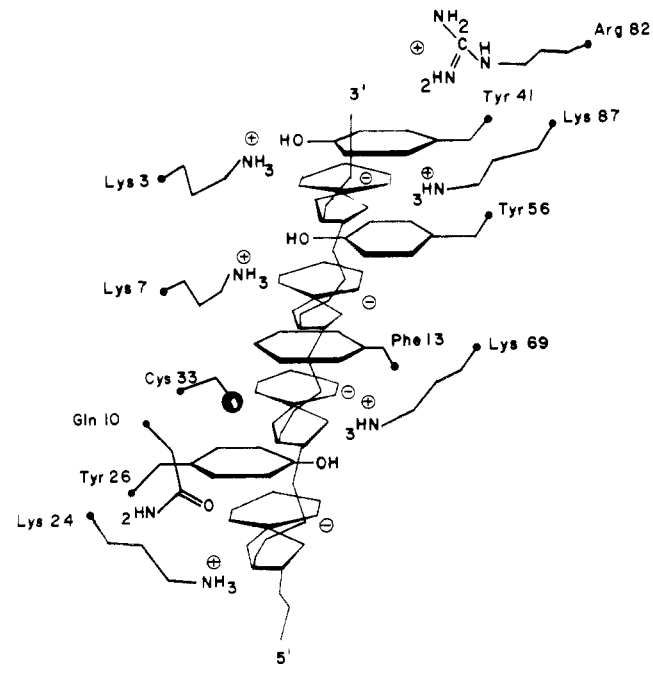

FIGURE 8: Model of the DNA binding surface of gene 5 protein based on NMR and chemical-modification data.

The groups of protons undergoing clearly definable base-dependent upfield chemical shifts in the nucleotide complex are those of the phenylalanyl residue (Figures 5–7). Thus, intercalation of the Phe ring seems clear. On the other hand, from the proton data alone, definition of the exact nature of the shifts induced in the resonances of the 3,5-tyrosyl protons on complex formation is more difficult, since these resonances are broadened and overlap with the resonance of the H(1′) of the sugars (peak 10, Figure 4B).

While there is more resonance in the area of peak 10 of the 2,6-deuterated complex (Figure 7B), showing some of the resonance of the 3,5-tyrosyl protons to be near 6.3 ppm in the complex, the resonances of the 3,5-tyrosyl protons appear to be severely broadened in the complex, making quantitative assessment of the shifts of the 3,5-tyrosyl protons difficult based on the proton data alone. ^{19}F NMR of *m*-fluorotyrosyl residues specifically assays the environment of the positions normally occupied by the 3,5 protons of the tyrosyl residues and, hence, can be used to help define the chemical nature of the tyrosyl interaction. Early data using tetranucleotides of random sequence to form complexes with the ^{19}F-labeled protein had suggested that upfield "ring-current" chemical shifts were induced in the fluorine resonances of the surface tyrosyls by nucleotide binding (Anderson et al., 1975; Coleman et al., 1976). The use of homogeneous tetra- and octanucleotides to form the complexes with the ^{19}F-labeled protein defines these shifts more precisely. The ^{19}F resonances of two of the three surface fluorotyrosyls undergo upfield shifts of ~0.4 ppm for thymine-containing nucleotides and ~0.7 ppm for adenine-containing nucleotides (Figure 2, Table I). This is compatible with intercalation of two of the three tyrosyl residues. In contrast to the earlier spectra on complexes with random tetranucleotides, the ^{19}F NMR spectra of both d(pT)$_4$ and d(pA)$_8$ complexes resolve the resonance of one of the surface tyrosyls from the other two and show it to move slightly downfield (Figure 2). While intercalation of all three surface tyrosyls is shown in the model in Figure 8, the precise intercalation of one of them may be incorrect and involve some other sort of interaction. Ring-current shifts in fluorine could, however, be counteracted by simultaneous downfield shifts due to the removal of solvent and increased van der Waals contacts. On the other hand, the latter may be the only interaction with one of the tyrosyls.

Changes in protein conformation, in addition to the specific base–protein interactions, may accompany nucleotide binding. One indication of such changes may be the small downfield chemical shifts for the ^{19}F resonances of the buried tyrosyls on nucleotide binding, especially in the presence of the more tightly bound d(pA)$_8$ (Figure 2C). Although participation of arginyl residues in nucleotide binding has yet to be studied by chemical modification, ^{1}H NMR shows the resonance from the δ-CH$_2$ of at least one of the arginyl residues to undergo either a large chemical shift or extreme broadening on complex formation (Coleman et al., 1976). From the previous model, Arg-82 is in a position to interact with the nucleotide (Figure 8). While present evidence suggests that the positively charged amino acid side chains are involved directly in nucleotide binding, the specific residues shown in Figure 8 are speculative and are those close to the DNA binding surface in the previous model (Coleman et al., 1976).

As outlined under Results, the ^{19}F{^{1}H} NOE can potentially reveal much about internal motion of fluorotyrosyl residues in proteins. The NOE of *m*-fluorotyrosyl gene 5 suggests that the three "surface" residues involved in nucleotide binding have increased internal motion compatible with a model placing them on a DNA-binding surface in contact with the solvent. Further relaxation and NOE studies in D$_2$O and with deu-

terated fluorotyrosyl residues in the presence and absence of nucleotides should reveal more details of the tyrosyl–solvent, tyrosyl–nucleotide interactions.

Acknowledgment

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References

Alberts, B. M., and Frey, L. (1970), *Nature (London) 227,* 1313.
Alberts, B. M., Frey, L., and Delius, H. (1972), *J. Mol. Biol. 68,* 139.
Anderson, R. A., Nakashima, Y., and Coleman, J. E. (1975), *Biochemistry 14,* 907.
Chou, P. Y., and Fasman, G. D. (1974), *Biochemistry 13,* 222.
Coleman, J. E., and Armitage, I. M. (1977) in *NMR in Biology* (Dwek, R. A., Campbell, I. D., Richards, R. E., and Williams, R. J. P., Eds.) p 171, Academic Press, New York, N.Y.
Coleman, J. E., Anderson, R. A., Ratcliffe, R. G., and Armitage, I. M. (1976), *Biochemistry 15,* 5419.
Day, L. A. (1973), *Biochemistry 12,* 5329.
Griffiths, D. U., Feeney, J., Roberts, G. C. K., and Burgen, A. S. V. (1976), *Biochim. Biophys. Acta 446,* 479.
Hoult, D. I., and Richards, R. E. (1975), *Proc. R. Soc. London, Ser. A, 344,* 311.
Hull, W. E., and Sykes, B. D. (1975), *J. Mol. Biol. 98,* 121.
Mazur, B. J., and Model, P. (1973), *J. Mol. Biol. 78,* 285.
Noggle, J. H., and Schirmer, R. E. (1971), *The Nuclear Overhauser Effect, Chemical Applications,* p 73, Academic Press, New York, N.Y.
Oey, J. L., and Knippers, R. (1972), *J. Mol. Biol. 68,* 125.
Pratt, D., Laws, P., and Griffith, J. (1974), *J. Mol. Biol. 82,* 425.
Pretorius, H. T., Klein, M., and Day, L. A. (1975), *J. Biol. Chem. 250,* 9262.
Salstrom, J. S., and Pratt, D. (1971), *J. Mol. Biol. 61,* 489.

Structural Features of the α-Type Filaments of the Inner Root Sheath Cells of the Guinea Pig Hair Follicle†

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ABSTRACT: Since the protein filaments of the mature cells of the inner root sheath of the guinea pig hair follicle are cross-linked by the isopeptide bond ϵ-(γ-glutamyl)lysine, they may only be released from the tissue by brief trypsinization. The filaments are obtained in 60% yield (dry wt), are long tubes 70–80 Å in diameter, and contain citrulline, cross-link and 37% α helix. While this isolation procedure necessarily inflicts some damage, on dissociation two proteins were recovered from the filaments in 60% yield which had molecular weights of 52 000–56 000, contained 45–50% α helix, 28% of the total citrulline but only traces of cross-link. These proteins are probably fragments of the filament subunit(s), but estimates of the numbers or sizes of the intact subunits have not been possible. Further trypsin digestion of isolated filaments released two α-helix-enriched fragments which contained little citrulline and no cross-link. The smaller, particle 2, had a molecular weight of 40 000, an α-helix content of 85%, and dimensions of 170 × 20 Å and accounts for all of the α-helix of the filaments. It consists of a family of molecules each containing three chains of molecular weight 13 000–14 000 aligned side by side which presumably adopt a coiled-coil conformation. This coiled-coil segment or discrete region in the filaments is responsible for the α-type X-ray diffraction pattern given by the filaments. The larger, particle 1, had a molecular weight of about 100 000, contained 71% α helix, had dimensions of 400 × 20 Å, and can also account for all of the α-helix of the filaments. It contains three chains of molecular weight 32 000–35 000 and consists of two segments like particle 2 separated by a region of nonhelix. This particle may represent a major portion of a three-chain unit of the inner root sheath filament. Since the isolated filament proteins and α-helix-enriched particles contain only minor amounts of the citrulline and cross-link of the filaments, it is concluded that the majority of the citrulline and cross-link are located in regions of nonhelix on the filament subunits which are cleaved during the release of the filaments from the inner root sheaths.

The hair follicle is a complex structure in which several distinctly different cell types develop from a common cell population (Fraser et al., 1972). The most prominent are the cell types which comprise the rising column of the hair fiber (fiber cuticle, cortex, and in many coarser hairs the medulla). External to these is a concentric sheath of cells, the inner root sheath. This structure does not emerge from the surface of the skin with the growing hair and is degraded by proteolytic enzymes in the pilary canal (Gemmell & Chapman, 1971). The inner root sheath cells differentiate at an early stage and become filled with a fibrous protein that perhaps derives from (Rogers, 1964a) or admixes with (Parakkal & Matoltsy, 1964) an amorphous protein, trichohyalin. The terminally differentiated inner root sheath cells are packed with the fibrous pro-

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