Acyl Carrier Protein from *Escherichia coli:* Characterization by Proton and Fluorine-19 Nuclear Magnetic Resonance and Evidence for Restricted Mobility of the Fatty Acid Chain in Tetradecanoyl-Acyl-Carrier Protein†

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ABSTRACT: The acyl-carrier protein (ACP) of *Escherichia coli* is a protein of molecular weight 8847 with a 4′-phosphopantetheine prosthetic group. ACP functions (via the SH of the prosthetic group) as a coenzyme in the synthesis of fatty acids and complex lipids. We report proton nuclear magnetic resonance (NMR) studies of the structure of ACP under various experimental conditions. The motion of the fatty acyl chain of acyl-ACP has been investigated by ^{19}F NMR studies of difluorotetradecanoyl-ACP. ^{31}P NMR studies of the prosthetic group phosphorus of ACP and acyl-ACP are also reported. We make the following conclusions: (1) The structure of ACP is stabilized by surface charge, and (2) the fatty acid residue of acyl-ACP does not move freely and seems immobilized by an interaction with the protein moiety.

The acyl-carrier protein (ACP[1]) of *Escherichia coli* is a small, very acidic protein (M_r 8847), the amino acid sequence of which has been determined (Vanaman et al., 1968). Its prosthetic group, 4′-phosphopantetheine, is attached to serine-36 through a phosphodiester linkage (Prescott and Vagelos, 1972; Vanaman et al., 1968). ACP in its native state has been reported to have the properties of a typical globular protein with a low frictional coefficient and a considerable α-helical content (Takagi and Tanford, 1968). Despite numerous attempts, ACP has not been crystallized; thus, no X-ray diffraction data are available. ACP serves as the carrier of the intermediates in the fatty acid and phospholipid synthesis and interacts in a highly specific manner with at least 12 different enzymes (Prescott and Vagelos, 1972; Ray and Cronan, 1976b; Schulz et al., 1969).

This protein contains only four aromatic amino acids in its polypeptide chain of 77 amino acid residues (phenylalanine-28 and -50, histidine-75, and tyrosine-71) and is, therefore, an excellent protein for study by ^1H nuclear magnetic resonance (NMR) techniques. In addition, ACP can be labeled biosynthetically with 3-fluorotyrosine (Anderson et al., 1975; Hull and Sykes, 1974), and the environment of this residue can be studied by ^{19}F NMR.

The fatty acid chain in acyl-acyl-carrier protein (acyl-ACP[1]) is bound by a thioester linkage to the prosthetic group. Acyl-ACP is a key intermediate in the synthesis of phospholipids and it can be synthesized by a recently discovered enzyme called acyl-ACP synthetase (Ray and Cronan, 1976).

This enzyme ligates the fatty acid to the prosthetic group of the ACP. This method is, in contrast to chemical methods, absolutely specific, does not affect the native conformation of the protein (Ray and Cronan, 1976), and, thus, allows study of the structure of acyl-ACP.

In the paper, we present an analysis of the ^1H NMR spectrum of ACP with particular attention to the assignment of resonances and motions of the aromatic amino acids. The effect of pH and temperature on the structure of ACP has been investigated. ACP labeled with 3-fluorotyrosine has been used to study the position and motion of this residue. Finally, we present preliminary evidence for an interaction of the fatty acid chain with the protein in acyl-ACP labeled with 6,6- and 13,13-difluorotetradecanoic acids.

Materials and Methods

ACP was prepared from *E. coli* K12 essentially as described by Majerus et al. (1969). 3-Fluorotyrosyl-ACP was isolated in the same way from an *E. coli* K12 tyrosine auxotroph grown on 3-fluorotyrosine as described by Anderson et al. (1975). Both proteins were 99% pure as judged by ^1H nuclear magnetic resonance, amino acid analysis, and sodium dodecyl sulfate–acrylamide gel electrophoresis (Ray and Cronan, 1976). The 6,6- and 13,13-difluorotetradecanoic acids (prepared as described in Gent et al., 1976) were ligated to the ACP by utilizing the *E. coli* enzyme activity reported and characterized by Ray and Cronan (1976). The reaction was performed and the product purified as described by Ray and Cronan (1976) with the modifications of Spencer et al. (1978). The acyl-ACP produced by this method has exactly the same amino acid composition as pure ACP.

Samples for ^1H NMR measurements were heated in D_2O at 60 °C for 2 h to exchange labile protons, freeze-dried, and redissolved in D_2O. The final protein concentration was 1 to 3 mM in about 200 mM potassium phosphate buffer.

^1H NMR spectra were recorded in the Fourier mode on a Bruker HX-270 spectrometer equipped with a variable-temperature unit. All temperatures are uncorrected instrument readings. The solvent D_2O was used as the field-frequency lock. Irradiation of the proton resonance of water was carried out

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[1] Abbreviations used are: ACP, acyl-carrier protein; acyl-ACP, acyl-acyl-carrier protein; DSS, 4,4-dimethyl-4-silapentanesulfonic acid.

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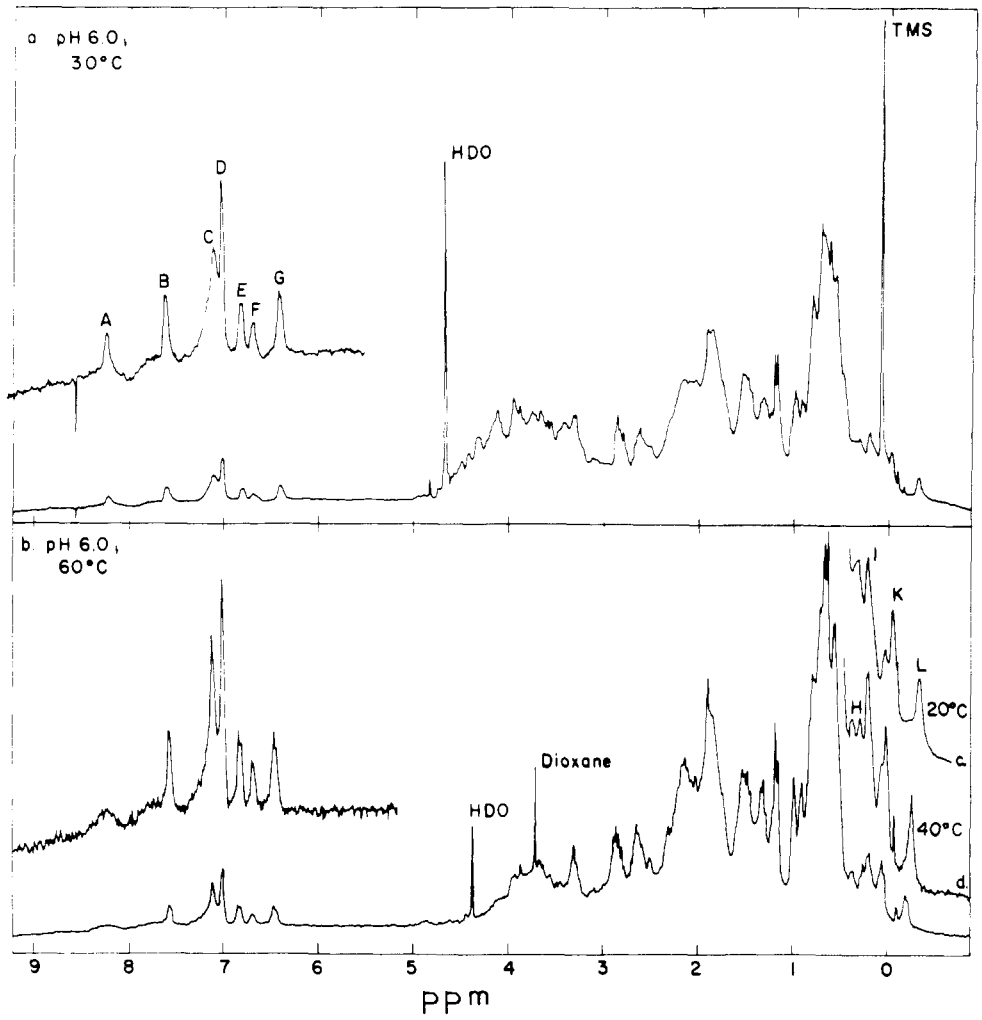

FIGURE 1: The 270-MHz ^1H NMR spectrum of native ACP in ~200 mM potassium phosphate buffer of pH 6.0 at 30 (a) and 60 °C (b); the high-field region at 20 (c) and 40 °C (d). External references are TMS in CCl_4 and dioxane. The HDO signal is repressed by the gated decoupling technique.

by the gated decoupling technique. Either dioxane or tetramethylsilane in carbon tetrachloride were used as external standards. All chemical shifts are quoted in parts per million (ppm) downfield from sodium 4,4-dimethyl-4-silapentanesulfonate (DSS).

^{19}F NMR spectra were recorded on an extensively modified Bruker HFX-90 single-coil pulsed Fourier transform spectrometer at a ^{19}F frequency of 84.67 MHz (Gent et al., 1976). A deuterium lock was used for field-frequency stabilization, and chemical shifts are reported in parts per million relative to neat external CF_3COOH. All measurements were done at 25 °C. Spin–lattice relaxation times (T_1) were determined by the inversion–recovery method, and proton decoupling was used where indicated.

^{31}P NMR spectra were recorded on the same spectrometer operating at 36.4 MHz at 25 °C. External D_2O was used for the field-frequency lock, and all spectra were obtained under conditions of proton noise decoupling. Chemical shifts are reported in parts per million relative to external 85% H_3PO_4.

Results and Discussion

The Overall Structure of ACP as Observed by ^1H NMR. The 270-MHz ^1H NMR spectrum of native acyl-carrier protein at pH 6.0 and 30 °C (after the exchange of all labile protons by D_2O) is shown in Figure 1a. The insert on the left side represents a magnification of the aromatic region between 6- and 9-ppm downfield from DSS. The structure of the spectrum is typical for native, globular proteins. The spectrum shows many overlapping lines between 0.5 and 4.5 ppm, a better resolved aromatic region, and some resonances of methyl and methylene groups around 0 ppm shifted by the local ring-current field of neighboring aromatic amino acids or other effects (Wagner and Wüthrich, 1975). Numerous resonances for protons were seen at low field when the sample examined had been exchanged against D_2O at room temperature. This suggests that there are regions in the protein not readily accessible to water.

The low-field resonances of the aromatic amino acids are clearly resolved and they must account for the corresponding 16 protons of the rings of the four aromatic amino acids phenylalanine-28 and -50, histidine-75, and tyrosine-71. The resonances can be assigned as follows: The single proton resonance at 8.21 ppm (A) must be due to the C(2)H proton of histidine-75, because it shifts to considerably higher field if the pH is raised from 6.0 to 9.6 (Table I). Histidine is the only amino acid with a pK in this region. The less deshielded C(4)H proton of histidine-75 can be seen at 7.10 ppm at pH 6.0 (C); this peak shifts to 6.80 ppm at pH 8.4 (Table I, Figure 2) and is obviously a one-proton resonance.

Furthermore, there are two resonances at 6.79 (E) and 6.39 ppm (G) corresponding to two protons each. At high temperature and pH each signal shows a splitting of about 8 Hz. The chemical shifts and splitting of these peaks are characteristic

TABLE I: pH Dependence of the Chemical Shifts in the Aromatic Region in ACP.

pH	6.0	7.7	8.4	9.0	identification
A	8.21	8.00	7.74	7.63	His-C(2)H
B	7.59	7.67	7.63	7.63	Phe
C	7.10	7.12	6.80	6.74	His-C(4)H
D	7.01	7.08	7.07	7.07	Phe
E	6.79	6.90	6.90	6.92	Tyr(2,6)
F	6.67	6.76	6.73	6.75	Phe
G	6.39	6.55	6.57	6.61	Tyr(3,5)

for the 2,6 (E) and 3,5 (G) protons of the tyrosine ring showing an AA'BB'-type spectrum. Such a spectrum can result from fast flipping on the NMR time scale of the aromatic ring around the C_β–C_γ bond (Wagner and Wüthrich, 1975). This assignment is further supported by the spectrum of a mixture of about 80% 3-fluorotyrosyl-ACP and 20% unmodified ACP (Figure 2c). The intensities of resonances E and G are clearly correspondingly reduced, with the 3-fluorotyrosine residue giving rise to a complex, unresolved ^1H spectrum in the region of resonance F.

The remaining peaks in the aromatic region are a two-proton peak at 7.59 ppm (B), a one-proton resonance at 6.67 ppm (F), and several overlapping peaks between 7.0 and 7.2 ppm (D). Resonances B and F can be explained only if we assume that one phenylalanine residue gives rise to an AA'BB'C-type spectrum whereby the remaining two-proton peak is in the region 7.0 to 7.2 ppm.

The structure of ACP was then examined as a function of temperature and pH. At pH 6.0, ACP is stable up to 60 °C and shows only minor changes in its ^1H NMR spectrum (Figure 1). The mobility of the side chains of the amino acid residues increases with rising temperature, as shown by the sharpening of the peaks in all regions. However, the overall three-dimensional structure of ACP is not altered significantly, as indicated by the lack of change in chemical shifts. The only readily observable changes are shifts of less than 0.2 ppm of some resonances in the high field and the aromatic region (Figure 3). The ring-current field is intrinsically independent of temperature (Dwek, 1973). If we assume that the high-field resonances are shifted mainly by this effect, we can conclude that the average distance between the rings and these methyl and methylene groups increases and that the structure becomes somewhat looser with rising temperature.

The conformation of ACP at pH 7.7 (Figure 4a) seems to be quite different from that at pH 6.0 (Figure 1a). The fine structure of the entire spectrum changes to some extent and all aromatic resonances absorb at different frequencies (Table I). The stability of the protein is diminished at this pH. ACP loses its three-dimensional structure between 40 and 50 °C and shows a spectrum typical of an aggregated denatured protein at 50 °C (Figure 4b) with little fine structure in the high-field resonances and degeneration of the aromatic resonances. The spectra at pH 6 (Figure 1) show little change between 30 and 60 °C. Circular dichroism spectra of ACP at pH 7.6 also demonstrate a change from a highly structured conformation with a considerable α-helical content at low temperature (<40 °C) to a random-coil conformation at high temperature (>50 °C; Schulz, 1977). ACP is a very acidic protein, and it was shown in the same paper (Schulz, 1977) that the addition of divalent cations stabilizes the conformation of ACP against thermal denaturation. It is interesting to notice that a lowering of the pH also stabilized the protein conformation. These findings suggest that a decrease of the high surface charge of the acidic protein increases its stability and may even trigger

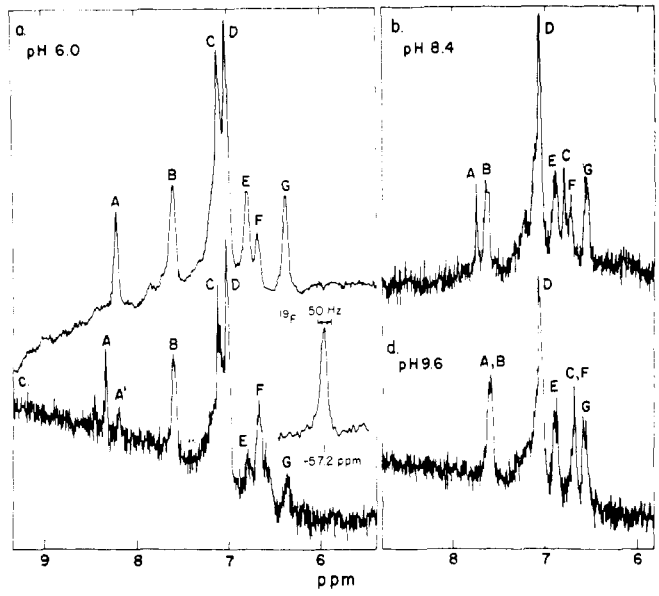

FIGURE 2: The aromatic region of the 270-MHz ^1H NMR spectrum of ACP in ~200 mM potassium phosphate buffer of pH 6.0 (a), 8.4 (b), and 9.6 (d) at 25 °C. (c) The aromatic region of a mixture of ACP (~20%) and 3-fluorotyrosyl-ACP (~80%) at pH 6.0 and 25 °C. The inset in c shows the 84.67-MHz ^{19}F NMR spectrum of this mixture under the same conditions.

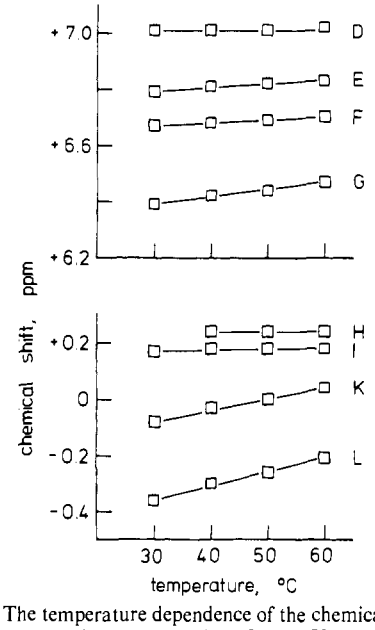

FIGURE 3: The temperature dependence of the chemical shifts of the high field and the aromatic resonances in ACP at pH 6.0. Designations refer to Figure 1 and Table I.

conformational changes. Between pH 7.7 and 9.6 the structure of ACP changes little and the only significant shifts are due to the titration of histidine-75 (Table I and Figure 2).

Location of the Tyrosine Residue of ACP. In order to examine the environment of the single tyrosine residue of ACP, the ^{19}F spectrum of ACP containing 3-fluorotyrosine was examined. The undecoupled 84.67-MHz ^{19}F NMR spectrum obtained at pH 6.0 and 25 °C is shown in Figure 2c (inset). The single resonance located −57.2 ppm from trifluoroacetic acid has a line width of about 30 Hz and must correspond to the single tyrosine-71 residue of ACP. In the ^1H NMR spectrum of native ACP, the protons of position 3 and 5 of the tyrosine ring are shifted about 0.5-ppm downfield from the random-coil position. This effect is presumably due to ring-current shifts.

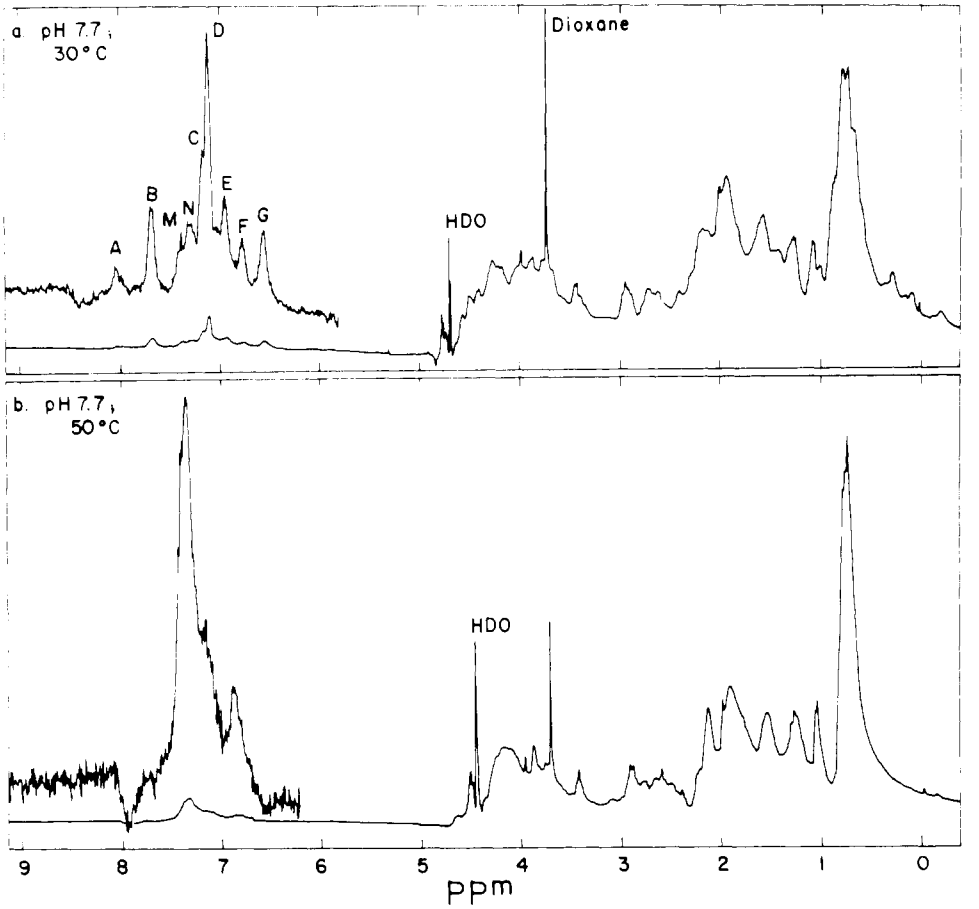

FIGURE 4: The 270-MHz ^1H NMR spectrum of ACP in ~200 mM potassium phosphate buffer of pH 7.7 at 30 (a) and 50 °C (b).

If we assume that this effect shifts the ^{19}F NMR resonance by the same amount, we can calculate the chemical shift of the ^{19}F NMR resonance due to polar, van der Waals, and other effects to be −57.7 ppm. This chemical shift is not significantly downfield relative to that of free 3-fluorotyrosine (−58.0 ppm), and based on the interpretation of Hull and Sykes (1975b) one would conclude that the tyrosine residue is solvent exposed. This finding is in conflict with the chemical modification studies of Abita et al. (1971), who reported that the tyrosine residue of ACP was abnormally resistant to nitration by tetranitromethane. Both methods are susceptible to error; ^{19}F NMR resonances can reflect local electric fields and hydrogen bonding as well as the more general environmental polarizability. By the same token, several proteins of known structure have tyrosine residues which are exposed to the solvent but which are, nonetheless, resistant to nitration (Glazer, 1976). Surface exposure of tyrosine-71 must remain in question.

We have also measured the spin-lattice relaxation time T_1 of the ^{19}F nucleus in 3-fluorotyrosyl-ACP by using the inversion-recovery technique. Proton decoupling could not be used because of the negative nuclear Overhauser enhancement (NOE) observed. It has been shown, however, that a single fluorine in a multispin proton system like a protein will relax with essentially a single time constant even when the proton system is not saturated (Hull and Sykes, 1975a). Under this condition, we measured a spin-lattice relaxation time T_1 of 150 ms. The nuclear Overhauser enhancement (NOE), the fractional increase or decrease in intensity of the fluorine resonance due to the saturation of the proton resonances (μ), was determined to be −0.7. The values of the T_1 and NOE values are in accord with the dominance of the dipole-dipole relaxation mechanism (Hull and Sykes, 1975b), and the rotational correlation time (about 10^{-8} s, Gerig, 1977) calculated for ACP.

Structure of Acyl-ACP. To study the structure of acyl-ACP, we prepared tetradecanoyl-ACP labeled with fluorine in the fatty acid chain. This chain is covalently bound as a thioester to the 4′-phosphopantetheine moiety that is itself attached to the serine-36 residue of ACP through a phosphodiester linkage. At 25 °C and pH 6.0, two resonances at −18.2 and −17.0 ppm from trifluoroacetic acid can be seen in the 84.67-MHz ^{19}F NMR spectrum of 6,6-difluorotetradecanoyl-ACP (Figure 5a). If the protein is denatured by the addition of 6 M guanidine hydrochloride (Takagi and Tanford, 1968), the signals merge and we observe only a single broad resonance (Figure 5b) at −17.2 ppm. This finding indicates that there is only one type of fluorine-containing group in the sample. The ^{19}F NMR spectrum of acyl-ACP labeled with 13,13-difluorotetradecanoic acid shows, however, only one broad resonance with a line width of about 80 Hz at −10.0 ppm. The spin-lattice relaxation time T_1 of this group is 0.36 ± 0.05 s.

Although of a preliminary nature, these spectra indicate a substantial and differential immobilization of the fatty acid moiety. If the fatty acid chain displayed no geometrically well-defined interaction with the protein but were completely free and unhindered, we would expect to observe a single sharp resonance for the difluoromethylene group. The rapid rotations of the chain around the carbon-carbon single bonds would lead to an equivalent environment for the two fluorines. A single sharp resonance for a *gem*-difluoro group is seen, for example, in the ^{19}F NMR spectra of 6,6-difluorotetradecanoic acid in chloroform (Gent et al., 1976) and in aqueous micellar dispersion.

The fact that we observe two resonances of equal height and

width for 6,6-difluorotetradecanoyl-ACP can be explained in two different ways. One is that there are two conformations for acyl-ACP with equal energy and, therefore, equal populations. However, there is no supporting evidence for two conformations and two specific equally populated binding sites are rather unlikely on such a small hydrophilic protein (neither gel filtration nor sedimentation analyses of ACP-SH and of acyl-ACP show any evidence of aggregation, even at protein concentrations >4 mM; C. O. Rock and J. E. Cronan, Jr., manuscript in preparation). A more plausible explanation is that the two fluorine atoms are magnetically nonequivalent due to the specific interaction of the protein with the fatty acyl chain, the two observable resonances being the strongest lines of an AB quartet. Given the size of geminal fluorine coupling constants (~200 Hz), the outer lines of the quartet would indeed be sufficiently weak to be lost in the noise.

The shift to a single line, 0.4-ppm downfield from the average fluorine environment upon denaturation in guanidine hydrochloride, is at least consistent with removing the chain from a hydrophobic binding site. Aqueous micelles of 8,8-difluorotetradecanoic acid in which adjacent chains provide a hydrophobic environment give a resonance at −23.7 ppm. This resonance shifts downfield 2.4 ppm on dilution to a point below the critical micelle concentration (M. -J. Lias and J. H. Prestegard, unpublished data). It should be noted that the downfield rather than the upfield shift upon removal from a hydrophobic environment is opposite to that seen for fluorotyrosine residues (Hull and Sykes, 1975b). This suggests that the direction of the shift is as much a property of the molecular probe as of the environment in which it is found.

The fact (Figure 5c) that the 13,13 derivative gives a broad resonance with a relatively short T_1 is consistent with a protein–fatty acid interaction extending over most of the length of the chain. The width is most likely the result of partially resolved nonequivalence of the two fluorines. The resonance position at −10 ppm is consistent with a slightly hydrophobic environment if the shift is corrected for an inherent 7.7-ppm difference in the resonance position of 13,13- and 6,6-substituted acids, as observed in chloroform solution (L. -d. Ong and J. H. Prestegard, unpublished data).

The presence of difluoro groups on the fatty acid moiety of acyl-ACP could, in principle, perturb the structure of acyl-ACP. However, the perturbation, if any, must be small, since *E. coli* is able to incorporate large amounts of these acids into the cellular phospholipids (Gent et al., 1978). The results must be taken as evidence of a fairly general fatty acid chain binding site on ACP.

^{31}P NMR was also used to study the effect of this covalent modification on the diester phosphate of the 4-phosphopantetheine moiety. The ^{31}P chemical shifts relative to 85% H_3PO_4 for ACP-SH and acyl-ACP at pH 8.0 were −0.13 and −0.96 ppm, respectively, and the line width at half peak height in both cases was approximately 12 Hz. No change in the chemical shifts was observed on lowering the pH to 5.2 (as expected for a diester phosphate). The chemical-shift difference of 0.83 ppm is significant for phosphate resonances; however, the exact nature of the alterations in the phosphate group is not known.

Conclusions

The ^{1}H NMR spectrum of ACP is typical of that seen for globular proteins, and the aromatic region is readily analyzable. The high-field resonances reflect small changes in the conformation as a function of temperature. ACP is more stable at pH 6.0 than at pH 7.7, which can be explained by a decrease in surface charge. The NMR results are indicative of the tyrosine-71 residue being exposed to solvent and not enclosed in the protein structure. Although this is not a definitive test of surface exposure, it brings to question previous conclusions based on resistance to nitration. The fatty acids of acyl-ACP do not move freely, and their hindrance can be attributed to an interaction of the fatty acid with the protein moiety.

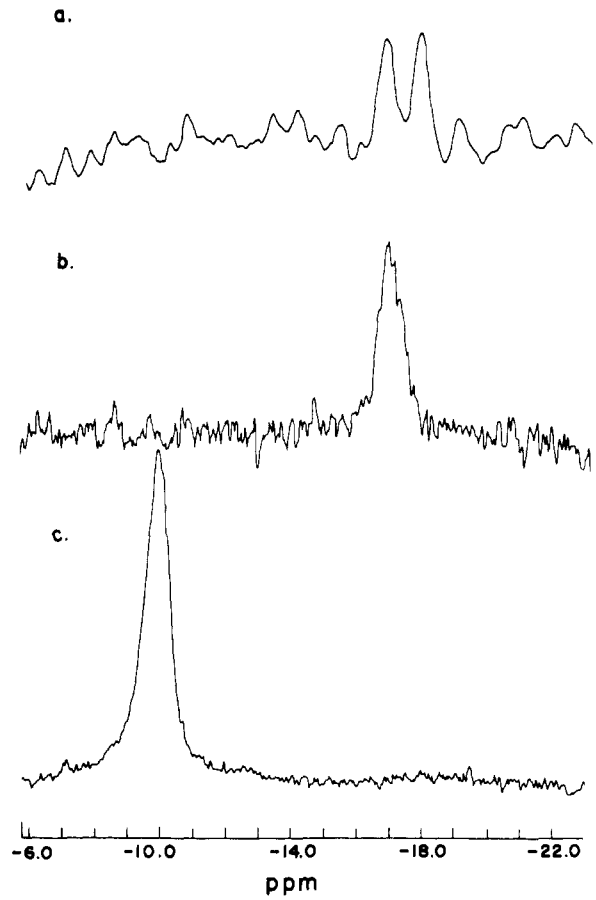

FIGURE 5: The 84.67-MHz ^{19}F NMR spectrum of difluorotetradecanoyl-ACP in ~200 mM potassium phosphate buffer of pH 6.0 at 25 °C: (a) 6,6-difluoromyristoyl-ACP in D_2O, (b) the same compound in the presence of 6 M guanidine hydrochloride, (c) 13,13-difluoromyristoyl-ACP in D_2O.

Acknowledgments

The acyl-ACP samples used for the ^{31}P spectra were synthesized by Dr. Charles O. Rock.

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Effects of Polyoxyanions on Sulfhydryl Group Modification of Thymidylate Synthetase†

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ABSTRACT: The reactivity of the catalytic cysteines of thymidylate synthetase in the presence and absence of polyoxyanions was studied by inactivation of the enzyme with methyl methanethiolsulfonate (MMTS) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). When measured in various buffers, the rate of the MMTS-dependent inactivation decreased in the series Tris-Cl > Tris-acetate > piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) > Tris-AsO_4 > Tris-SO_4 > phosphate > Tris-PO_4 and the analogous series for DTNB-dependent inactivation is Tris-Cl > Tris-SO_4 > phosphate > Tris-phosphate. This buffer dependence of the rates is interpreted in terms of a polyoxyanion interaction with the enzyme. MMTS inactivation of thymidylate synthetase was characterized by incorporation of 1.5-1.6 mol of [^{14}C]CH_3S- per mol of enzyme dimer using [^{14}C]MMTS, with concomitant loss of both enzymatic activity and ability to form covalent ternary complexes. Protection by pyrimidine derivatives and folates in the presence and absence of polyoxyanions was also investigated. The pH dependence of MMTS inactivation of Pipes buffer is in qualitative agreement with our earlier results in phosphate (Munroe, W. A., et al. (1978) *Biochem. Biophys. Res. Commun. 80,* 355-360). Observation of a finite reaction at low pH is consistent with activation of the catalytic cysteines by an as yet unidentified general base.

Thymidylate synthetase isolated from amethopterin resistant *Lactobacillus casei* catalyzes the reductive methylation of dUMP to form dTMP employing the coenzyme CH_2H_4folate.[1] This reaction is believed to proceed through a transient covalent ternary complex involving nucleophilic addition of a catalytic cysteine to carbon 6 of the uracil ring and attachment of the methylene portion of the coenzyme to carbon 5 of the uracil ring (see Danenberg, 1977, and references therein). This information has been inferred from studies of the stable covalent ternary complex composed of the enzyme, coenzyme, and the substrate analogue FdUMP. Recent ^{19}F NMR studies have delineated the relative stereochemistry of the ternary complex in the active site of the enzyme (Byrd et al., 1977, 1978; Byrd, 1977). The role of the catalytic cysteines has been further elaborated by correlation of extent of thiol modification with loss of enzyme activity and ability to form ternary complexes (Plese & Dunlap, 1977). Our recent studies have focused on the activation of the catalytic cysteine residues through interaction with an as yet unidentified general base residue in the active site as demonstrated by the pH dependence of the rate of inactivation of thymidylate synthetase by MMTS and DTNB (Munroe et al., 1978).

In order to further our understanding of the activation of the cysteine residues, we have extended our studies of MMTS inactivation of thymidylate synthetase. We have investigated the protection from MMTS inactivation of the enzyme by substrates and products and their analogues under a variety of conditions and have determined the stoichiometry of inactivation by this reagent. During these studies we observed that the presence of polyoxyanions had a pronounced effect upon the rate of inactivation and the degree of protection afforded by the substrate dUMP. In view of the observed effect we have extended our studies of the pH dependence of MMTS inactivation of thymidylate synthetase to include Pipes buffer which appears to be a noninteracting buffer.

Materials and Methods

Thymidylate synthetase was purified in the presence of exogenous thiols from amethopterin resistant *Lactobacillus*

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[1] Abbreviations used: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); dUMP, deoxyuridylate; dTMP, deoxythymidylate, FdUMP, 5-fluorodeoxyuridylate; dCMP, deoxycytidylate; dU, deoxyuridine; (±)-CH_2H_4folate, (±)-5,10-methylene-5,6,7,8-tetrahydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; MMTS, methyl methanethiolsulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol.

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