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Biosynthesis and Characterization of [^{15}N]Actinomycin D and Conformational Analysis by Nitrogen-15 Nuclear Magnetic Resonance†

Richard H. Shafer,* Joseph V. Formica, Claudio Delfini,‡ Stephen C. Brown, and Peter A. Mirau§

ABSTRACT: We describe the production and characterization of actinomycin D labeled with ^{15}N at all twelve nitrogen positions. Cultures of *Streptomyces parvulus* were incubated in the presence of racemic [^{15}N]glutamic acid and, following an initial delay, labeled antibiotic was produced. Evidence is presented that the D enantiomorph of glutamic acid was ultimately used for actinomycin biosynthesis. The ^{15}N NMR spectrum at 10.14 and 20.47 MHz of the labeled drug in $CDCl_3$ is presented. All nitrogens except the phenoxazone chromophore nitrogen are inverted when spectra are obtained under broad-band proton irradiation conditions. All ^{15}N resonances have been assigned, and the proton-nitrogen one-bond coupling constants were determined in $CDCl_3$ to be 92.5 ± 0.3 Hz for the valine and threonine amide protons by both ^{1}H and ^{15}N NMR. ^{15}N NMR spectra were also obtained in dimethyl sulfoxide, methanol, and water in order to probe solvent interactions with the peptide nitrogens and carbonyl groups. Large downfield shifts (>5 ppm) were seen for the Pro, sarcosine, and methylvaline resonances when the solvent was changed from dimethyl sulfoxide to water. Smaller downfield shifts were observed for the Val and Thr peaks. These results are discussed in terms of a model for the solution conformation of the actinomycin pentapeptide rings based on different hydrogen-bonding interactions in the monomer in organic solvents and the dimer which is formed in water.

Actinomycins are peptide-containing antibiotics isolated from cultures of various species of *Streptomyces*. Actinomycin D (ACTD, Figure 1),[1] the most extensively studied actinomycin, is of biological significance as a potent inhibitor of RNA synthesis and as a clinical anticancer agent. In addition, the complex of ACTD with DNA serves as a model for studies of protein-nucleic acid interactions. These various properties of actinomycins have been discussed in several recent reviews (Meienhofer & Atherton, 1977; Mauger, 1980).

Investigations directed toward studying controlled biosynthesis of actinomycins have resulted in the production of many congeners (Mauger, 1980; Meienhofer & Atherton, 1977; Formica & Apple, 1976) which have been used effectively as probes to study RNA synthesis and the mode of binding to DNA and as putative cancer chemotherapeutic agents (Müller

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[1] Abbreviations: ACTD, actinomycin D; Me_2SO, dimethyl sulfoxide; NOE, nuclear Overhauser effect.

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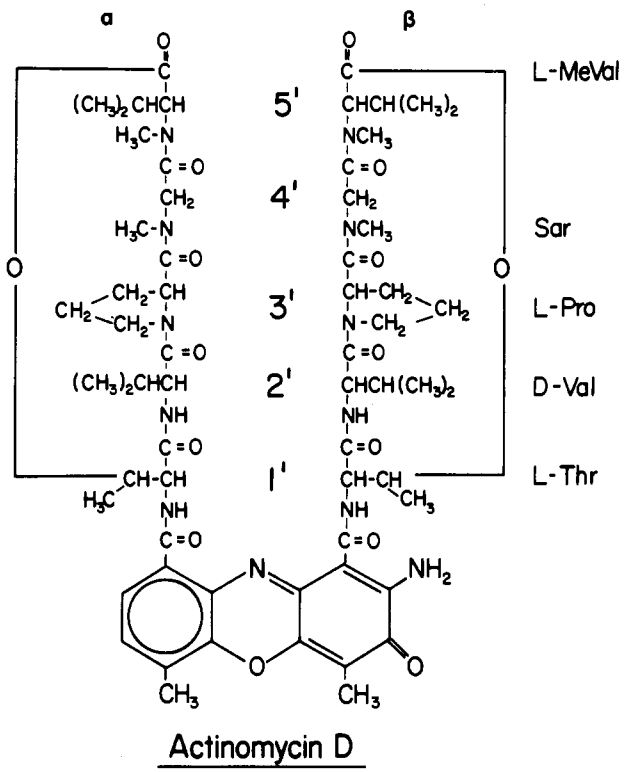

FIGURE 1: Structure of actinomycin D. The peptide lactone ring attached to the benzoid end of the chromophore is the α-pentapeptide while the other is the β-pentapeptide.

& Crothers, 1968; Meienhofer & Atherton, 1977; Shafer et al., 1980; Mirau & Shafer, 1982a). Consistent with this interest to produce useful biochemical probes, the synthesis of ACTD labeled with ^{15}N was undertaken. Incorporation of sufficient levels of this isotope should permit ^{15}N NMR studies of ACTD and its complex with DNA. *Streptomyces parvulus* was the species of choice as it produces virtually only ACTD. Glutamate was used as the sole source of nitrogen because previous studies showed that this amino acid was most efficiently utilized for actinomycin production (Katz et al., 1958).

The ready availability of racemic [^{15}N]glutamic acid necessitated preliminary studies to determine the effect of the D enantiomorph on growth and production. Previous studies with D-valine, D-isoleucine, or D-alloisoleucine established that the presence of these amino acids in the culture medium inhibited production of actinomycins (Katz, 1960). Similarly, the addition of D-pipecolic acid to the medium substantially diminished production of the pipecolic actinomycins (May & Formica, 1974). The experiments reported below describe the biosynthesis and isolation of ^{15}N-labeled ACTD and its characterization by ^{1}H and ^{15}N NMR.

The presence of ^{15}N at high levels of enrichment provides an opportunity to apply ^{15}N NMR to the problem of conformational analysis of ACTD in solution. Efforts in this area have been predominantly focused on proton NMR and have been reviewed in detail by Lackner (1975). Analysis of the three-dimensional structure of actinomycins has been profoundly influenced by the X-ray crystal structure obtained by Sobell and co-workers (Sobell et al., 1971; Jain & Sobell, 1972) of ACTD complexed with deoxyguanosine. We report below the ^{15}N NMR spectrum of [^{15}N]ACTD in Me_2SO, methanol, and water as well as $CDCl_3$ in an effort to probe the degree of solvent exposure of amide protons and carbonyl groups. The results of this study suggest a different conformation for ACTD in organic solvents, and possibily in the monomeric form in water, than that described by Lackner (1975).

Materials and Methods

Chemicals, Reagents, and Isotopes. DL-[^{15}N]Glutamic acid (95 atom % ^{15}N) was purchased from Merck Sharp & Dohme Canada, Ltd., D- and L-glutamic acids were purchased from Sigma Chemical Co., and reagent-grade D-fructose was obtained from Fisher Scientific Co. Reagents for the determination of L-glutamate were available in food analysis kit no. 139092 from Boehringer Mannheim Biochemicals. Unlabeled ACTD was obtained from Sigma Chemical Co. Me_2SO-d_6 was purchased from Wilmad Glass Co.; $CDCl_3$ and D_2O were obtained from Aldrich Chemical Co. Unlabeled NMR solvents were analytical reagent grade. The other chemicals were of the highest quality available from commercial sources.

Conditions of Cultivation. Streptomyces parvulus (American Type Culture Collection 12434) was obtained from Dr. Edward Katz of Georgetown University, Washington, DC, on glucose–yeast extract–malt extract–agar slants. For propagation of the culture, the spores were scraped gently from the surface of the slant and used to inoculate NZ amine medium (Katz & Gross, 1959). After being incubated for 2 days at 28 °C in a gyrotary shaking incubator, the mycelium was harvested by centrifugation, suspended in saline, and used to inoculate sterile soil supplemented with 0.25% dried blood and 1.0% calcium carbonate. The soil cultures were incubated at room temperature for 5 days, after which they were stored at 4 °C.

The vegetative inoculum was prepared by transferring approximately 25 mg of the soil culture to 100 mL of NZ amine medium and incubating as described. After a 48-h incubation at 28 °C, the cultures were harvested and washed by centrifugation and then resuspended in 50 mL of saline. Aliquots of this suspension served as inoculum for the chemically defined production medium.

Analytical Procedures. Growth of the organism was determined by collecting the mycelium by vacuum filtration on tared Whatman no. 1 filter paper which was then dried at 105 °C to constant weight. Actinomycin production was monitored spectrophotometrically at 442 nm. Samples were removed at the intervals indicated, extracted with an equal volume of ethyl acetate, and assayed. The product was identified as actinomycin D by circular paper chromatography (Formica & Katz, 1973; May & Formica, 1974; Formica & Apple, 1976). Residual L-glutamate was measured enzymatically with the Boehringer Mannheim L-glutamate dehydrogenase food analysis kit, using the aqueous fraction of the ethyl acetate extracted medium.

Production and Isolation of [^{15}N]*ACTD.* The medium for the production of ACTD consisted of 40 g of D-fructose, 1.0 g of K_2HPO_4, 25 mg each of $ZnSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and $MgSO_4 \cdot 7H_2O$, 2.1 g of glutamic acid, and 1000 mL of deionized water, pH 7.1. The glutamic acid was added as the D and/or L isomer to a final concentration of 14 mM. Production medium was inoculated and incubated as described. After 12 days of incubation, the cultures were harvested by filtration, and the filtrate was extracted with an equal volume of ethyl acetate. After the extract was evaporated to dryness, the residue was purified by preparative descending paper chromatography (Formica & Katz, 1973; May & Formica, 1974; Formica & Apple, 1976). ACTD was isolated by extracting the appropriate area on the chromatograms and subjecting the extract to further purification by using silicic acid column chromatography (Formica & Katz, 1973).

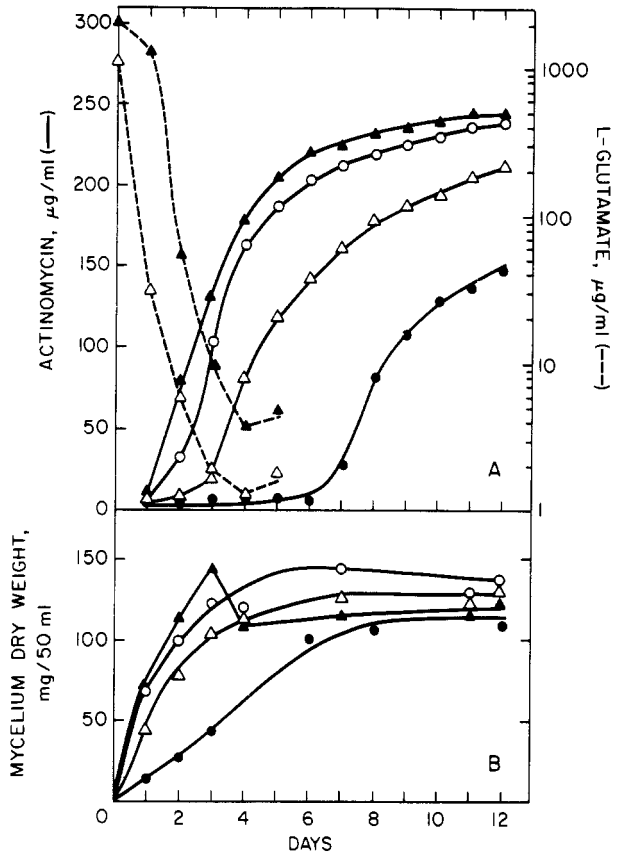

FIGURE 2: Effect of D- and/or L-glutamate on actinomycin production (A), growth of *S. parvulus* (B), and uptake of L-glutamate (---). (▲) 2.1 g of L-glutamate/L; (○) 0.525 g of D-glutamate plus 1.575 g of L-glutamate per L; (△) 1.05 g of D-glutamate plus 1.05 g of L-glutamate per L; (●) 2.1 g of D-glutamate/L.

NMR. Proton spectra were obtained at 100 MHz on a Varian XL-100 spectrometer equipped with a Nicolet Fourier-transform accessory. Proton spectra were also obtained at 240 MHz by using a spectrometer constructed by Drs. V. Basus and J. Murphy in the UCSF School of Pharmacy NMR Facility. ^{15}N spectra were obtained at 10.14 MHz on the Varian XL-100 spectrometer and at 20.47 MHz on the UCB200 spectrometer at the University of California, Berkeley Chemistry Department NMR Facility. All spectra were obtained by using an internal or external deuterium lock. ^{1}H spectra were referenced to residual solvent and reported relative to Me_4Si, and ^{15}N chemical shifts were assigned by referencing to an external standard of neat formamide.

Results

Effect of Contrived Mixtures of Glutamate on Growth and Production. Production media were prepared with glutamate at a final concentration of 14 mM. Cultures were sampled daily for determination of growth and antibiotic production. The data listed in Figure 2 reveal that in media containing only L-glutamate, production and growth were initiated within 24 h, as expected. As the amount of D-glutamate was increased in the contrived racemic mixtures, initiation of production and growth was delayed. In the presence of D-glutamate alone, antibiotic production was delayed for 6 days with growth following similar kinetics. After 12 days of incubation, the culture in which 25% of the glutamate was in the D form showed inhibition of production by only 1%. Cultures containing 50% and 100% of glutamate as the D enantiomorph showed 14% and 40% inhibition of production, respectively (Table I).

Table I: Effect of D- and/or L-Glutamate on Actinomycin Production and Growth of *Streptomyces parvulus*

glutamic acid (g/L)		% of control[a]	
D	L	growth	production
0	2.1	100	100
2.1	0	91	61
0	1.575	83	75
0.525	1.575	108	99
0	1.05	55	50
1.05	1.05	102	86

[a] Based on values obtained from the 12th day of incubation.

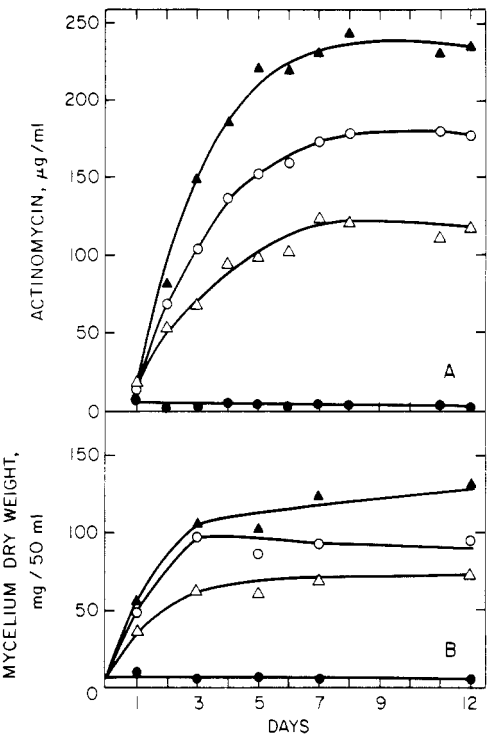

FIGURE 3: Effect of L-glutamate on actinomycin production (A) and growth of *S. parvulus* (B). (▲) 2.1 g of L-glutamate/L; (○) 1.575 g of L-glutamate/L; (△) 1.05 g of L-glutamate/L; (●) no glutamate.

Media were prepared in which only the L isomer was used at final concentrations of 14, 10.5, 7.0, and 0 mM in order to demonstrate that decreased production was not due solely to the availability of the L isomer of glutamate. These concentrations reflected the amount of L isomer used in the contrived racemic mixtures in the prior experiment. The data shown in Figure 3A reveal that production was not delayed and that after 12 days of incubation production was inhibited 0, 25, 50, and 100%, respectively. Growth of the organism behaved in a similar manner (Figure 3B). These values are consistent with the percent reduction in concentration of the nitrogen source in the medium (Table I).

The higher yield of antibiotic in the presence of the D isomer suggests that the organism has the ability to utilize the unnatural isomer. In addition, L-glutamate disappeared exponentially during the first 4 days of incubation in culture containing 100% L- and 50% DL-glutamate (Figure 2A). Moreover, L-glutamate was not detected over the 12-day incubation period in samples taken from cultures which contained only D-glutamate. After the fourth day of incubation, the amount of L-glutamate present, if any, was outside the limits of detection by the assay employed.

Examination of the products of these incubations by circular paper chromatography revealed that the products were iden-

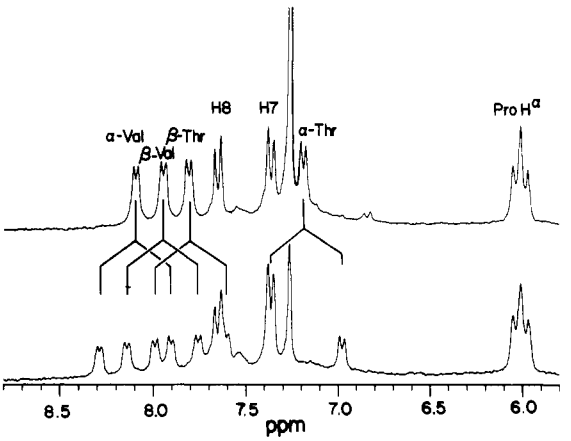

FIGURE 4: Low-field portion of the ^{1}H NMR spectra of ACTD (top) and [^{15}N]ACTD (bottom) in $CDCl_3$ (10 mM) at 240 MHz. In each case, 600 transients were acquired with a 2-s recycle time.

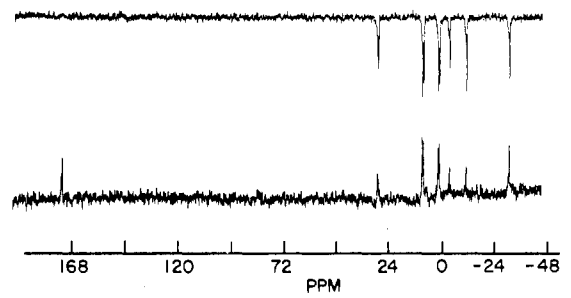

FIGURE 5: ^{15}N NMR spectra at 20.47 MHz of [^{15}N]ACTD (17 mM) at 22 °C in $CDCl_3$: with broad-band decoupling and NOE, 332 transients (top); with gated decoupling to suppress the NOE, 1000 transients (bottom). Spectra were obtained with a 45° pulse, a 1.2-s acquisition time, and a 5-s delay time.

tical and corresponded to authentic actinomycin D. The yield in all cases was 90–92% ACTD, the rest being distributed among three chromatographically slower moving components (data not shown).

^{1}H NMR. The low-field ^{1}H NMR spectra at 240 MHz of ACTD and [^{15}N]ACTD in $CDCl_3$ are presented in Figure 4. This region contains the valine and threonine amide protons, each appearing as a doublet due to coupling to the corresponding H$^{\alpha}$ proton. Assignment of peaks to the α- or β-peptides was achieved by Lackner (1971) using selective deuteration. In the labeled sample, each amide proton is split further by one-bond coupling to ^{15}N. The absence of any significant intensity at frequencies expected for unlabeled ACTD provides a measure of the high level of labeling achieved at the valine and threonine nitrogens.

^{15}N NMR. The ^{15}N NMR spectrum of [^{15}N]ACTD at 20.47 MHz in $CDCl_3$ is shown in Figure 5. The spectrum obtained with the nuclear Overhauser effect (NOE) shows six resonances while the spectrum obtained without NOE displays all seven resonances. The farthest downfield peak in the latter spectrum is due to the phenoxazone nitrogen and was always nulled under NOE conditions. All other peaks were inverted when spectra were obtained with NOE, due to the negative gyromagnetic ratio of the ^{15}N nucleus. Figure 6 presents ^{15}N NMR spectra at 10.14 MHz with the NOE with and without proton decoupling. As can be seen in both Figures 6 and 7, three resonances (proline, valine, and threonine) appear split due to the magnetic nonequivalence of the α- and β-pentapeptide rings.

Assignment of the ^{15}N resonances was accomplished by analogy with model compounds, proton decoupling, and selective ^{15}N{^{1}H} NOE experiments. The data in Figure 6 reveal

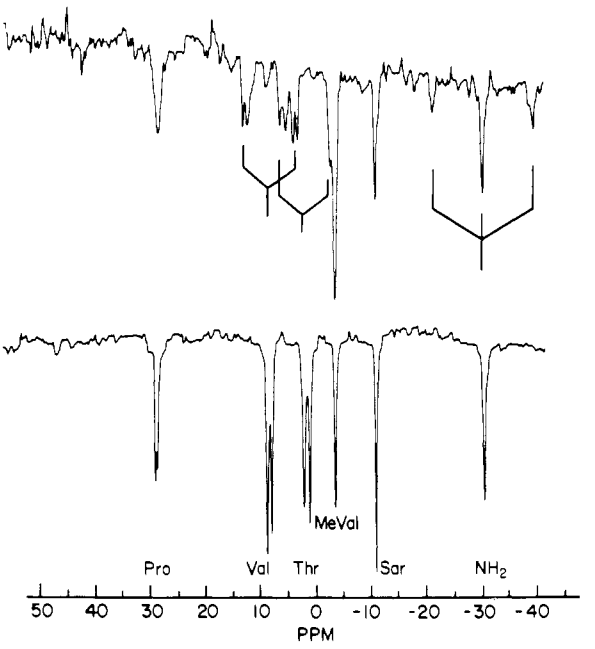

FIGURE 6: ^{15}N NMR spectra at 10.14 MHz of [^{15}N]ACTD (12 mM) at 15 °C in $CDCl_3$ with broad-band decoupling and NOE (bottom) and with gated decoupling to retain the NOE and coupling (top). Spectra were obtained in 600 transients with a 45° pulse, a 1.5-s acquisition time, and a 0.5-s delay time.

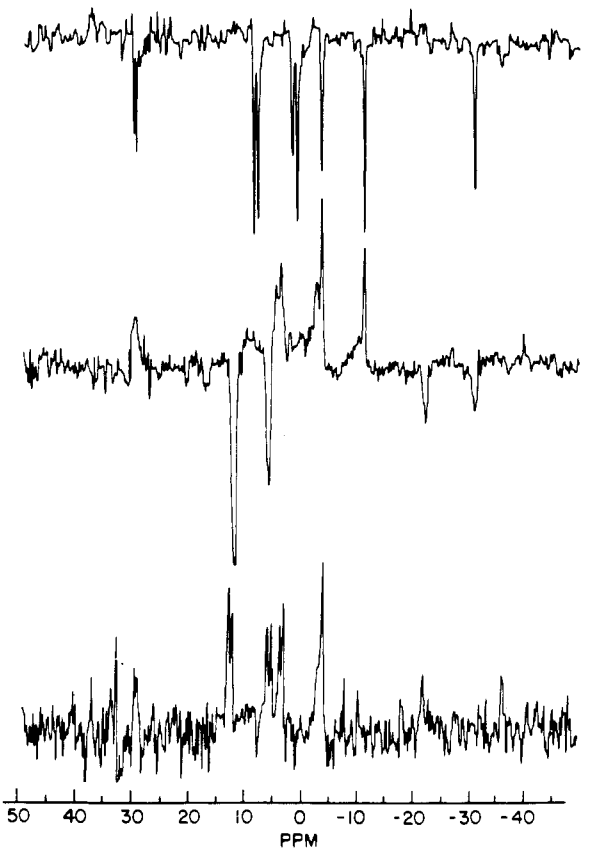

FIGURE 7: Selective NOE spectra of [^{15}N]ACTD (10 mM) in $CDCl_3$: with broad-band decoupling and NOE, 600 transients (top); with selective irradiation downfield from the Val NH protons (middle); and with selective irradiation of the low-field Sar H$^{\alpha}$ resonance (bottom). Selective NOE spectra were obtained by using 15 000 transients.

that the only resonance appearing as a triplet in the coupled spectrum is the highest field one at −30.4 ppm, which must be the amino nitrogen attached to the chromophore. This

Table II: ^{15}N Chemical Shifts for [^{15}N]ACTD in Various Solvents at 15 °C[a]

	chemical shift (ppm)					
solvent	Val	Thr	Pro	Sar	MeVal	-NH_2
Me_2SO	7.0	1.9, 1.8	29.1	−10.7, −10.9	−2.5	−26.4
methanol	7.6, 6.5	1.9, 1.2	30.4, 29.8	−8.2	−2.0	−30.5
water	8.9, 8.0	5.0, 4.8	34.5	−3.9	3.8	−33.2
$CDCl_3$	8.4, 7.8	1.6, 0.9	29.4, 28.9	−10.9	−3.4	−30.4

[a] ^{15}N chemical shifts referenced to external neat formamide at 15 °C. Downfield shifts with respect to this reference are positive.

upfield position is typical of amines (Levy & Lichter, 1979). The phenoxazone ring nitrogen was assigned to the furthest downfield resonance at 172.4 ppm in the bottom spectrum of Figure 6, based on the large deshielding typical of pyridine-like nitrogens and the nulling of the resonance when NOE is applied.

The spectrum obtained by gated decoupling (Figure 6) shows that the two doublets at 8.4 and 7.8 ppm and at 1.6 and 0.9 ppm arise from singly protonated nitrogens which must correspond to the valine and threonine resonances. Assignment of these resonances was made by selective $^{15}N\{^1H\}$ NOE experiments, the results of which are shown in Figure 7. In the ^1H NMR spectrum of ACTD in $CDCl_3$, the furthest downfield resonance has been assigned to the α-valine (Figure 1) amide proton (Lackner, 1971). Irradiation was carried out downfield from the α-valine proton in order to avoid affecting the nitrogen of β-threonine, whose amide proton resonates close to the β-valine amide proton. As seen in Figure 7, this produced an inversion of the nitrogen resonance that occurs near 8 ppm. Furthermore, this negative resonance is only partially decoupled due to the off-resonance conditions. This firmly assigned the doublet at 8 ppm to the valine nitrogens and, consequently, the doublet near 1 ppm to the threonine nitrogens.

Assignment of the sarcosine and methylvaline resonances was carried out in a similar fashion. Selective irradiation of one of the sarcosine H^α protons produced the bottom spectrum shown in Figure 7. While no inversion of any resonances occurred, the peak at −10.9 ppm showed a substantial loss of intensity, approaching that of the noise. Thus, this resonance is assigned to sarcosine. As this nitrogen is not directly bonded to a proton, it is likely that both the methylene and methyl protons contribute to its relaxation. Consequently, it is expected that irradiation of only the former would give rise to a partial NOE, as observed.

Finally, selective irradiation of the proline H^α protons also resulted in the doublet at 29.4 and 28.9 ppm in Figure 6 decreasing to the level of noise (data not shown), assigning it to the proline nitrogens. This then leaves the peak at −3.4 ppm in Figure 6 arising from methylvaline, thereby completing the assignments.

The 10.14-MHz ^{15}N NMR spectra of [^{15}N]ACTD in Me_2SO, methanol, and water are displayed in Figure 8, and the results are summarized in Table II. Peak assignments were made on the basis of those determined in $CDCl_3$ and on measurements in binary solvent mixtures of varying composition. All of the peptide resonances showed downfield shifts when the solvent was changed from Me_2SO to methanol to water. The amino resonance showed an upfield shift upon transfer from Me_2SO to methanol to water. This sequence of solvents is characterized by increasing ability to donate a proton to a hydrogen bond acceptor moiety. Thus, these results can be used to probe the extent of solvent exposure of the peptide bond, as discussed below.

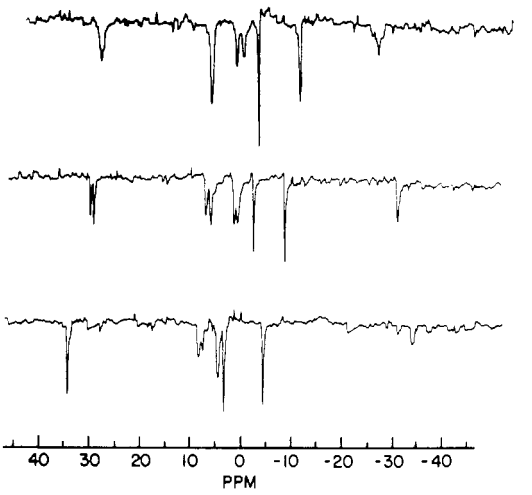

FIGURE 8: ^{15}N NMR spectra of [^{15}N]ACTD at 15 °C with broadband proton decoupling and NOE: 12 mM in 86% Me_2SO–14% Me_2SO-d_6, 600 transients (top); 12 mM in MeOH, 600 transients (middle); 3.7 mM in 90% H_2O–10% D_2O, pH 6, 3600 transients (bottom). All spectra were obtained with a 1.46-s acquisition time and a 3.5-s delay time.

One obvious difference in the spectra shown in Figures 6 and 8 is the effect of solvent on the multiplicity of the peaks arising from the Pro, Val, and Thr resonances. These peaks are resolved into doublets in both $CDCl_3$ and methanol, but some appear as broad singlets in Me_2SO and/or water. These effects can be attributed to small differences in magnetic nonequivalence due to solvent interactions as well as differences in dynamics. Higher resolution spectra obtained at higher field strengths should elucidate the sources of such effects.

It is important to note that ACTD forms dimers in aqueous solutions (Müller & Emme, 1965; Müller & Spatz, 1965; Crothers et al., 1968; Angerman et al., 1972). At the concentration used in this study, the dimer is the predominant form. Hence, all of the data in water more appropriately refer to the dimer, rather than the monomer. We note that the chemical shifts in methanol showed no significant change upon titration with water until the concentration of water exceeded 93 mol %. Thus, the spectrum in methanol may closely approximate that of the ACTD monomer in water. The considerable reduction in signal intensity in water for the amino, valine, and threonine resonances is due to H–D exchange with D_2O in the 90% H_2O–10% D_2O mixture used in obtaining the aqueous spectrum.

Discussion

Biosynthesis of [^{15}N]*ACTD.* Apparently, *S. parvulus* is able to utilize D-glutamate as the sole source of nitrogen for growth and production of ACTD. The means by which this feat is accomplished is not clear. Conceivably, transamination between pyruvate (derived from the metabolism of fructose) and D-glutamate could result in the production of α-ketoglutarate and L-alanine. Subsequent utilization of these products supports growth and production. Alternatively, a specific or direct racemization of D- to L-glutamate could occur with subsequent utilization. The presence of such transaminases or racemases has not been reported for *S. parvulus* (Katz, 1960). In this study, L-glutamate was not detected in samples taken from media containing only D-glutamate. It can be speculated that glutamate remained undetected because

the substrate was internalized and converted, with little excess escaping into the culture fluid. The ability of L-glutamate to induce generalized transport has been reported previously (May & Formica, 1978).

The fact that ^{15}N was incorporated to a large extent in the product could indicate that the L isomer was used immediately for growth and that when the organism entered its idiophase, the D isomer, which was spared, is now racemized and utilized exclusively for the synthesis of antibiotic. In any event, the yields and high degree of enrichment with ^{15}N indicate that both isomers were utilized for the production of actinomycin D.

Characterization of [^{15}N]ACTD. ^{1}H NMR analysis of [^{15}N]ACTD provides some evidence of the high degree of labeling, and the ^{15}N NMR spectrum confirms the fact that ^{15}N has been substituted at all positions, with similar levels of enrichment. Mass spectrometric analysis by fast atom bombardment (unpublished data) shows that the pseudomolecular ion of the labeled drug is 12 amu higher than that of the unlabeled drug, with a 90–95% average level of isotopic enrichment, corroborating the NMR results. Both ^{1}H and ^{15}N NMR spectra provide independent determinations of the ^{15}N–^{1}H one-bond coupling constants for the valine and threonine residues, with $J_{^{15}N-^{1}H} = 92.5 \pm 0.3$ Hz in both cases by both methods in $CDCl_3$. Longer range coupling constants appear to be too small to be measured readily as often found in saturated systems (Binsch et al., 1964). It is interesting to note that the ^{1}H NMR spectrum of ACTD shows the major magnetic nonequivalence in the same three residues as the ^{15}N spectrum (Lackner, 1971; Mauger, 1980).

Solvent Exposure Studies. ^{15}N NMR has been used to characterize the solution conformation of several cyclic polypeptides, such as gramicidin S (Khaled et al., 1978; Hawkes et al., 1980), viomycin (Hawkes et al., 1978), alumichrome (Llinas et al., 1976; by indirect measurement of the ^{15}N spectrum), and others (Williamson et al., 1979; Live et al., 1979), as well as linear peptides (Kricheldorf et al., 1977; Hull & Kricheldorf, 1980). These studies have demonstrated that ^{15}N NMR is particularly well suited for probing the hydrogen-bonding and solvent-exposure properties of peptide nitrogens and carbonyls. Hence, we have pursued a similar approach in an effort to characterize the solution conformation of ACTD, in particular in aqueous solution.

The rather extensive data on the conformation of ACTD in various solvents have been summarized by Lackner (1975). ACTD appears to be a very rigid structure whose conformation remains essentially the same in organic solvents as well as in water. Thus, the apparent uniqueness of conformation justifies the interpretation of chemical shift changes in different solvents in terms of the degree of solvent exposure. Interpretation of the spectrum in water is aided by the model for the ACTD dimer proposed by Angerman et al. (1972) based on proton chemical shifts. This model consists of two ACTD molecules with their chromophores stacked together, the quinoid ring of one over the benzoid ring of the other, and vice versa. Proton chemical shifts on the peptide groups showed little or no dependence on concentration, and thus the peptide rings are assumed not to interact in dimer formation. The same pattern of proton shifts was also observed by Krugh & Neely (1973).

Examination of the spectra in Figure 8 and the chemical shift data in Table II reveals that all of the peptide resonances show downfield shifts when the solvent is changed from a hydrogen bond accepting solvent, Me_2SO, to a hydrogen bond donating solvent, water. The downfield shifts can be explained in terms of solvent effects at the amide bond nitrogen and carbonyl groups. Solvent interactions which result in electron delocalization lead to downfield shifts (Llinas et al., 1976; Khaled et al., 1978). Usually, donation of an amide proton to an acceptor solvent produces a smaller downfield shift than that resulting from donation of a solvent proton to an amide carbonyl (Hawkes et al., 1980).

Analysis of the N-substituted amino acids is facilitated by the absence of an amide proton, and hence, solvent perturbations are centered at the carbonyl of the adjacent residue sharing the peptide bond. Thus, the downfield shift of ~7 ppm for the sarcosine (Sar) resonance upon transfer from Me_2SO to H_2O reflects the hydrogen bond donating capacity of H_2O. The shift is somewhat larger than that seen by Kricheldorf et al. (1977) for polysarcosine undergoing the same change of solvent and indicates that the Pro carbonyl is exposed to solvent. The methylvaline (MeVal) and Pro resonances shift downfield by ~5–6 ppm when the solvent is changed from Me_2SO to H_2O, which suggests that the Sar and Val carbonyls are also exposed to solvent. Alternatively, these downfield shifts could indicate the presence of intramolecular hydrogen bonds in the water dimer that are not present in Me_2SO. These shifts are similar to those observed in gramicidin S for the valine nitrogen, whose amide proton participates in an intramolecular hydrogen bond, and for the proline nitrogen upon transfer from Me_2SO to trifluoroethanol (Khaled et al., 1978; Hawkes et al., 1980).

Solvent effects at the Val and Thr resonances may involve interactions at both the carbonyl and nitrogen positions. The chemical shifts for these peaks are in general smaller than those found for the N-substituted residues. This may reflect the overall effect of increasing proton-donating capacity of the solvent to the carbonyl with decreasing proton-accepting capacity of the solvent from the protonated nitrogen. Both proton NMR (Victor et al., 1969; Conti & De Santis, 1970) and IR (Von Dreele & Stenhouse, 1974) studies in chloroform indicate that the Val amide proton participates in an intramolecular hydrogen bond. Thus, one expects that the major effect on the Val nitrogen resonance is due to solvent interaction with the Thr carbonyl. Interestingly enough, this effect appears to be relatively small as the chemical shift changes upon solvent transfer to water are smallest for the Val peak, 1–2 ppm.

The Val N also shows very little change in chemical shift between Me_2SO and methanol. This resonance appears as a singlet in the former and a doublet in the latter, with an average chemical shift very close to that of the singlet. Thus, the magnetic nonequivalence of the Val resonances appears enhanced in methanol relative to Me_2SO. Due to this solvent-induced splitting, it is difficult to assess quantitatively the significance of solvent exposure at the Thr–Val bond. The effect, however, appears to be small.

The effect of changing the solvent from Me_2SO to water is seen to be more substantial for the Thr resonance, 3 ppm, potentially reflecting interactions at both the chromophore carbonyl and Thr NH groups. In organic solvents, the Thr NH proton resonance shows very little temperature dependence, as does the Val NH proton (Victor et al., 1969; Mirau & Shafer, 1982b). Also, Conti & De Santis (1970) have shown that in $CDCl_3$ the α-Thr NH proton requires a considerable time for exchange with D_2O. These results indicate that the Thr NH groups are somewhat inaccessible to solvent. The very small shifts for the Thr nitrogen upon transfer from Me_2SO to methanol are consistent with the proton NMR data. Furthermore, that one peak shifts upfield indicates an effect at the Thr nitrogen and hence that the chromophore carbonyl

groups are also solvent protected. Upon transfer to water, however, these groups appear significantly more exposed to solvent.

It is also of interest to compare our results for the individual Thr nitrogen resonances with the proton NMR studies of Arison & Hoogsteen (1970). They showed that the upfield Thr NH peak was consistently deshielded more than the downfield peak by proton-accepting solvents. Lackner (1971) subsequently assigned the downfield Thr NH proton to the β (quinoid) pentapeptide and the upfield one to the α (benzoid) pentapeptide. When a small amount of D_2O was added to [^{15}N]ACTD in $CDCl_3$, the Thr nitrogen at lower field disappeared rapidly (data not shown), assigning it to the β pentapeptide, in parallel with the Thr NH proton assignments. The data in Table II show that in the ^{15}N spectrum, it is the upfield or α-Thr resonance that suffers larger changes in the two organic solvents, in agreement with proton NMR results (Arison & Hoogsteen, 1970).

Finally, the chromophore NH_2 group shows a considerable upfield shift following transfer from Me_2SO to methanol or water, due to the decreasing hydrogen bond accepting potential of the latter two. In addition, ring-current effects arising from chromophore stacking in the water dimer may contribute to the amino nitrogen chemical shift.

In summary, the above analysis provides an indication of the degree of solvent exposure or hydrogen bonding of all peptide carbonyls except that of MeVal. Of these four, substantial downfield shifts (>5.4 ppm) are observed for the Pro, Sar, and MeVal nitrogens upon transfer from Me_2SO to water. In particular, the Val C=O appears to be sensitive to solvent perturbations and thus does not behave as if it were involved in an intramolecular hydrogen bond that persists in all the solvents studied. However, the currently accepted model for the solution conformation of ACTD (Mauger, 1980; Meienhofer & Atherton, 1977; Lackner, 1975), based on the ACTD–deoxyguanosine X-ray structure (Jain & Sobell, 1972), involves two hydrogen bonds from the Val C=O of each peptide ring to the Val NH of the other peptide ring. Such a model would predict little if any effect on the Pro nitrogen resonance in solvent titrations.

An alternative model consistent with the ^{15}N NMR results presented above and the X-ray crystal structure of ACTD in the ACTD–deoxyguanosine complex (Jain & Sobell, 1972) as well as the NMR and IR evidence for the participation of the Val NH in a hydrogen bond in organic solvents (Victor et al., 1969; Conti & De Santis, 1970; Von Dreele & Stenhouse, 1974) would involve a change in hydrogen bonding upon transfer from organic solvents, where ACTD is a monomer, to water, where ACTD is present as a dimer.

In the water dimer, formation of the two interpeptide Val NH to Val C=O hydrogen bonds would explain the downfield shift of the Pro N, the other downfield shifts arising from solvation of the corresponding carbonyl groups. Structurally, these interpeptide hydrogen bonds might function to keep the peptide groups of each partner in the water dimer away from each other.

The unresolved problem concerns the structure of the monomer in organic solvents as well as in water. The large downfield shifts observed for the Pro, Sar, and MeVal nitrogens suggest that neither the Val, the Pro, nor the Sar carbonyls are hydrogen bonded in the monomer. The ^{15}N NMR results do not provide any information concerning the MeVal carbonyl involved in the pentapeptide lactone ring closure. The possibility of a Val NH to MeVal C=O intrapeptide hydrogen bond was first proposed by Conti & De Santis (1970) but has been considered unlikely by De Santis et al. (1972) on the basis of their IR data, the overall rigidity of ACTD based on NMR line widths (Victor et al., 1969), and the relative insensitivity of CD spectra to changing solvent (Ascoli et al., 1972).

The possibility of a hydrogen bond from the Val NH to the phenoxazone C=O amide groups was also considered by De Santis et al. (1972). Such an interaction would be consistent with the very small changes we have observed in the Thr nitrogen chemical shift when the solvent is changed from Me_2SO to methanol (Table II). The 3-ppm downfield shift in the Thr resonance upon transfer to water from methanol may appear somewhat too large to be consistent with this model. However, consideration of the fact that the Thr NH is to some extent solvent protected in organic solvents suggests that interactions at both the Thr NH and the phenoxazone C=O may play a role in determining the Thr ^{15}N chemical shift in water. Thus, we cannot rule out completely the Val NH to phenoxazone C=O hydrogen-bonding scheme.

The most compelling argument based solely on the ^{15}N NMR results presented above can be made for the interpeptide Val NH to Thr C=O hydrogen bonds. This would explain the small ^{15}N chemical shift change observed for the Val resonances when the solvent is changed from Me_2SO to water (Table II). This scheme, however, was not even considered possible in the theoretical conformational analysis carried out by De Santis et al. (1972). To what extent such a structure could be accommodated remains to be seen.

One must also consider the possibility that the large shifts observed when the solvent is changed from Me_2SO to water are due to some major conformational change. If, as our data appear to indicate, the bulk of these changes in chemical shift, and hence the change in conformation, are associated with dimer formation, such a change would have to be consistent with the insensitivity to dimer formation observed for several pentapeptide proton resonances (Angerman et al., 1972; Krugh & Neely, 1973). Furthermore, temperature-jump studies have shown that dimer formation occurs on a fast time scale (Davanloo & Crothers, 1976). For these reasons, we presently favor a model that involves changes in hydrogen bonding without incurring a major change in conformation.

The experiments described above demonstrate the usefulness of ^{15}N NMR in the conformational analysis of ACTD. High levels of ^{15}N have been incorporated biosynthetically and permit NMR measurements on the millimolar concentration scale. Clearly, further work is needed to understand more fully the solution conformation of ACTD, in view of the data presented here based on ^{15}N NMR and other data published earlier. The full potential of ^{15}N NMR in this system, however, will be realized in studies with oligonucleotides and DNA.

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Aerobactin-Mediated Utilization of Transferrin Iron†

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ABSTRACT: Aerobactin and enterobactin, hydroxamate- and catechol-type siderophores, respectively, were found capable of removing iron(III) from transferrin in buffered solution. Although under these conditions aerobactin displaced the iron much more slowly than did enterobactin, the rate for the former could be accelerated by addition of pyrophosphate as mediator. Transfer of iron(III) from transferrin to aerobactin appeared to proceed via a ternary complex. Cells of *Escherichia coli* BN 3040 Nal^R *iuc* containing transport systems for both enterobactin and aerobactin, the genetic determinants for the latter specified on a ColV-type plasmid, took up iron from [^{55}Fe]transferrin in minimal medium. In this case aerobactin was effective at a much lower concentration, although enterobactin still displayed superior ability to transfer the iron. In serum, however, the rate measured with aerobactin exceeded that found with enterobactin. The results indicate that aerobactin, in spite of its relatively unimpressive affinity for iron(III) as a siderophore, is nonetheless equipped with structural features or properties that enhance its ability to remove the metal ion from transferrin, especially when receptor-bearing cells of *E. coli* are present to act as a thermodynamic sink for the iron. These attributes of the aerobactin system of iron assimilation may account for its status as a virulence determinant in hospital isolates of *E. coli*.

Aerobactin, the prototypical member of the hydroxamic acid–citrate family of siderophores, is a conjugate of 6-(*N*-acetyl-*N*-hydroxyamino)-2-aminohexanoic acid and citric acid. It is produced by *Aerobacter aerogenes*—in addition to the catechol-type siderophore enterobactin—under conditions of low iron stress. The high-spin octahedral iron(III)–aerobactin complex is formed with the two bidentate hydroxamate groups, the central carboxylate, and probably the citrate hydroxyl group. The stability constant for ferric aerobactin is many orders of magnitude below that of ferric enterobactin and approximates that of ferric transferrin (Harris et al., 1979). The question arises as to why the microorganism should produce the thermodynamically inferior chelator aerobactin when it can form the more powerful chelator enterobactin. Thus far there is no convincing answer to this question, though certain observations would tend to favor aerobactin as the more efficient chelator in a biological milieu.

Enterobactin is chemically unstable and is destroyed enzymatically following its iron transport function, whereas there is no evidence to show that aerobactin cannot be recycled. It

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