λ *cro* Repressor Complex with O_R3 DNA: ^{15}N NMR Observations†

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Received January 7, 1987; Revised Manuscript Received May 27, 1987

ABSTRACT: ^{15}N NMR studies of the coliphage λ *cro* repressor are presented. The protein has been uniformally labeled with ^{15}N, and individual amino acids have been incorporated. Although the four C-terminal residues (63–66) were not located in the original crystallographic studies of the protein [Anderson, W. F., Ohlendorf, D. H., Takeda, Y., & Matthews, B. W. (1981) *Nature (London) 290*, 754], it has been proposed that the C-terminus is involved in DNA binding [Ohlendorf, D. H., Anderson, W. F., Fisher, R. G., Takeda, Y., & Matthews, B. W. (1982) *Nature (London) 298*, 718]. These experiments give direct verification of that proposal. [^{15}N]Amide resonances are assigned for residues 56, 62, 63, and 66 in the C-terminus by enzymatic digestion and by ^{13}C–^{15}N double-labeling experiments. $^{15}N\{^1H\}$ nuclear Overhauser effects show that the C-terminus is mobile on a nanosecond time scale. Exchange experiments using distortionless enhancement via polarization transfer, which is sensitive to proton exchange on the $1/J_{NH}$ (10 ms) time scale, indicate that the amide protons in the C-terminus are freely accessible to solvent. It is thus a flexible arm in solution. The binding of both specific operator and nonspecific DNA is shown to reduce both the mobility and the degree of solvent exposure of this arm. Two-dimensional ^{15}N–^1H correlation experiments using ^{15}N-labeled *cro* reveal inconsistencies with previously reported ^1H NMR assignments for the lysine amides [Weber, P. L., Wemmer, D. E., & Reid, B. R. (1985) *Biochemistry 24*, 4553]. This result suggests that those assignments require reexamination, illustrating the utility of ^{15}N labeling for obtaining ^1H resonance assignments of biomolecules. Furthermore, isomerization of the peptide bond of Pro-59, which has been previously suggested (Weber et al., 1985) and which would significantly affect the properties of the C-terminal arm, is shown to not occur.

The *cro* repressor is a small, basic, dimeric protein (66 amino acids, M_r 7351 per subunit) that regulates the switch from lysogenic to lytic growth in λ phage (Ptashne, 1986). The X-ray crystal structure of *cro* repressor has been solved to 2.2 Å (Anderson et al., 1981), and on the basis of this structure, a model for its interaction with the operator O_R3 has been proposed (Ohlendorf et al., 1982) and recently refined (Hochschild & Ptashne, 1986). This model proposes a general motif for protein–DNA interactions, this being an α-helix–turn–α-helix which lies in the major groove of B-form DNA. This motif has been observed by X-ray crystallography in *cro* repressor, CAP (McKay & Steitz, 1981), the amino-terminal fragment of λ repressor (Pabo & Lewis, 1982), and *trp* repressor (Schevitz et al., 1985) and also by NMR[1] spectroscopy in the DNA-binding 51-residue amino-terminal fragment of *lac* repressor (Kaptein et al., 1985). Recent X-ray crystallographic studies of the complex of the phage 434 cI repressor amino-terminal domain with a 14 base pair operator fragment, solved to a resolution of 3.2 Å (Anderson et al., 1987), has confirmed the general features of this model.

In the crystal structure of *cro* repressor only residues 1–62 were located (Anderson et al., 1981); the remaining four residues were disordered. It was thus assumed that in solution these C-terminal four residues (and perhaps several others) form a mobile arm. Since this arm is both highly basic, containing two lysines, and seemingly suitably positioned to interact with the DNA phosphate backbone, in the building of the *cro*–DNA model the arm was placed in the minor groove of the DNA (Ohlendorf et al., 1982) (see also Figure 11). However, it is not known whether this arm does indeed form a mobile domain in solution, exactly how many residues form this domain, and whether it does or does not interact with DNA in the suggested manner.

Recently, Weber et al. (1985) assigned the proton NMR spectrum of *cro* repressor and on the basis of apparent split COSY peaks postulated that Pro-59 is in slow exchange on the NMR time scale between cis and trans isomers. Such an isomerization would move the C-terminal domain through a large region of space. This may have significant implications for the binding of *cro* to O_R3 and to its one-dimensional diffusion along the DNA.

In order to understand the dynamics of the C-terminal arm, the number of residues involved, and the possible role of proline isomerism, we undertook this study. As we shall show, these questions are particularly amenable to study by ^{15}N NMR spectroscopy.

The large size of the *cro*–O_R3 complex, ca. 25 kDa, gives a long correlation time and, accordingly, broad NMR resonances. These problems are minimized by use of nuclei of low gyromagnetic ratio, and thus we employed nitrogen-15 NMR.

Nitrogen has a large chemical shift range covering over 150 ppm for proteins. Furthermore, due to the low gyromagnetic ratio, ^{15}N NMR lines are substantially narrower than proton

† This research was supported by NIH Grant GM 32987 to P. Lu. The spectrometer was partially funded by NSF Grant PCM82-07163 to S. J. Opella and P. Lu.

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[1] Abbreviations: DEPT, distortionless enhancement via polarization transfer; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TSP, sodium 3-(trimethylsilyl)tetradeuteriopropionate.

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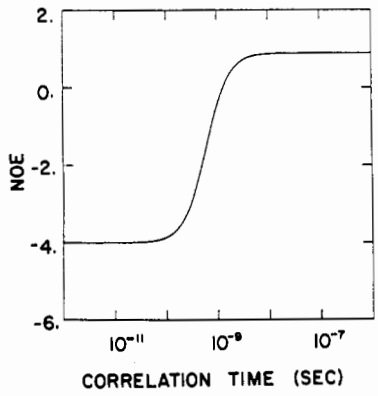

FIGURE 1: NOE ($1 + \eta$) vs rotational correlational time in seconds for ^{15}N{^{1}H} NOEs recorded at a field strength of 9.4 T, assuming isotropic reorientation for a two-spin (^{1}H–^{15}N) system relaxing exclusively by dipolar relaxation.

lines. The combination of these effects means that ^{15}N NMR spectra intrinsically offer higher resolution than proton spectra, typically giving in two-dimensional heteronuclear correlated spectra almost total resolution of all nitrogen sites for proteins of a few kilodaltons (Schiksnis et al., 1987).

The ^{15}N{^{1}H} nuclear Overhauser effect (NOE) may be used as a probe of the dynamics of the nitrogen site. The NOE enhancement ranges from −0.12 to −4.93 as a function of the rotational correlation time (Gust et al., 1975). This enhancement added to the original signal (+1.0) gives a net NOE that varies between +0.88 and −3.93, dependent upon the correlation time (see Figure 1). The rotational correlation time for most small- to medium-sized proteins (5000–25 000) falls in the range 2–30 ns (Cantor & Schimmel, 1980). Sites that are rapidly reorienting on the time scale of the Larmor frequency thus receive a large negative NOE, whereas sites that are rigid experience little or no enhancement.

The main disadvantages of ^{15}N for NMR studies are its exceedingly low natural abundance (0.37%) and its low gyromagnetic ratio ($\gamma_N \simeq \gamma_H/10$). It is for these reasons that relatively few ^{15}N NMR studies on proteins in solution have been reported [for example, Gust et al. (1975), Lapidot et al. (1976), Bogusky et al. (1985), LeMaster and Richards (1985), Smith et al. (1987), and Schiksnis et al. (1987)]. However, with uniform enrichment of the protein, obtained by overproduction on an expression vector, and by use of polarization transfer (Morris & Freeman, 1979; Doddrell et al., 1982; Pegg et al., 1982; Pegg & Bendall, 1983) and inverse detection techniques (Müller, 1979; Griffey et al., 1983; Bax et al., 1983; Live et al., 1984; Griffey et al., 1985), problems of sensitivity are reduced.

Materials and Methods

Isolation of Uniformly ^{15}N-Labeled cro Repressor. *cro* repressor was isolated by a variation of the method of Arndt et al. (1982). *Escherichia coli* strain GM1 (Miller et al., 1977) was transformed with a derivative of the plasmid pTR214, which was a gift from Tom Roberts. This plasmid, a pBR322 derivative (Bolivar et al., 1977), contains both the *cro* gene and the β-lactamase gene, with the *cro* gene being transcribed by the *tac* promoter (Maniatis et al., 1982). Typically, a 1-L culture of M9 media (Miller, 1972) prepared with 1 mg/mL [^{15}N]ammonium chloride or [^{15}N]ammonium sulfate and containing 40 mg/L ampicillin was innoculated and grown to an A_{550} of 0.9. Since the *cro* gene is under *lac* repressor control, it was induced by the addition of 10^{-3} M IPTG, with the simultaneous addition of a further 40 mg/mL ampicillin aliquot. Incubation was continued for 5 h, at which time the cells were harvested by centrifugation. The cells were lysed by hen egg white lysozyme and sonication, and the lysate was dialyzed against 20 mM potassium phosphate, 100 mM KCl, and 2 mM EDTA, pH 6.8, and loaded onto a 7-mL phosphocellulose column. *cro* repressor was eluted by a 40-mL linear gradient of increasing KCl concentration (0.1–1.0 M) and assayed by fluorescence (*cro* repressor contains only tyrosyl fluorophores) (Boschelli et al., 1982). The *cro* protein obtained in this manner was >95% pure, as judged by SDS–PAGE and fluorescence spectroscopy. The final yield was approximately 15 mg/mL of culture.

Isolation of [^{15}N]Ala-, [α,ϵ-$^{15}N_2$]Lys-, [α-^{15}N]Lys-, and [α-^{15}N]Lys,[1-^{13}C]Val-Labeled cro Repressor. The transformed *E. coli* strain GM1 was grown in 1 L of M9 media (Miller, 1972) containing 200 mg of all amino acids with the exception of the desired labeled species. The culture was induced as above, except that 100 mg of labeled L-amino acids (or 200 mg of DL) was also added at this time. The procedure was continued as described above, followed by gel filtration over Sephadex G-75 (buffer: 10 mM potassium phosphate, 50 mM KCl, 2 mM EDTA, pH 6.8) which gave >95% pure *cro* repressor when assayed as above. Under these conditions, *cro* repressor will approximately coelute from a G-75 column with soybean trypsin inhibitor (M_r 21 500) due to an equilibrium between dimeric and tetrameric states (Leighton and Cook, unpublished observations). The yield of ^{15}N-labeled *cro* repressor was similar to that obtained above.

DNA binding of the isolated proteins was independently assayed by the increase in the rotational correlation time of ethidium-labeled O_R3 fragments at pH 7.3 and 4.5 (Cook and Lu, personnal communication).

Sample Preparation. Uniformly ^{15}N-labeled *cro* repressor was dialyzed against 10 mM potassium phosphate, 200 mM KCl, and 10^{-5} M EDTA, pH 6.8 buffer to reduce the salt concentration. It was then concentrated by lyophilization and resuspended. [^{15}N]Ala- and [^{15}N]Lys-labeled *cro* repressors were lyophilized and resuspended.

After lyophilization, DNA binding was assayed by the quenching of tyrosyl fluorescence by O_R3 operator fragments (Boschelli et al., 1982); no reduction in the binding activity was found. The sample was then dialyzed either against 10 mM potassium phosphate, 0.2 M KCl, and 2 mM EDTA, pH 6.5, against 10 mM citrate, 0.2 M KCl, and 2 mM EDTA, pH 4.6, or against 0.2 M Tris-HCl, pH 8.5 (reported pHs are meter readings). A 1.8-mL sample of this solution was placed in a 10-mm NMR tube with 0.2 mL of D_2O added as an internal lock. For proton-detected experiments, 0.36 mL of sample solution and 40 μL of D_2O were placed in a 5-mm NMR tube, with a trace of TSP added as internal standard. When necessary, the pH was altered either by dialysis into fresh buffer or by the addition of small quantities of dilute HCl or KOH. All samples were 0.2–0.5 mM in *cro* repressor monomer, which was measured with a molar extinction coefficient of 3.97×10^3 at 280 nm (Boschelli et al., 1982).

O_R3 operator fragment, 17 base pair sequence corresponding to base pairs 37 951–37 967 inclusive of the λ sequence (Hendrix et al., 1983), was prepared by the solid-phase phosphite triester method on an Applied Biosystems 380A synthesizer. After cleavage of all protecting groups, the single strands were purified by ion-exchange HPLC (Partisil 10SAX column) with a linear phosphate gradient (1 mM to 0.4 M KH_2PO_4; formamide/water 50:50, pH 6.3). After being desalted over Sephadex G-10, the strands were combined in equimolar proportions as calculated from their respective ultraviolet absorbances at 260 nm. The solution was lyo-

philized to dryness and dissolved in appropriate buffer (as above), and the strands were annealed by heating to 100 °C and slow cooling. Nonspecific DNA fragments were prepared by digestion of calf thymus DNA (Sigma) with micrococcal nuclease (Sigma) (Boschelli et al., 1982). Fragments in the range 15–40 base pairs were pooled, desalted, and concentrated.

NMR Spectroscopy. ^{15}N NMR spectra were recorded on a JEOL GX 400WB NMR spectrometer operating in the Fourier transform mode at 40.508 MHz. Samples were kept at room temperature (~22–26 °C) for NMR measurements, since the protein solutions were stable at this temperature for the long periods required for data accumulation. Although other workers (Weber et al., 1985) have used sample temperatures of 30–35 °C for studying the proton spectrum of *cro* repressor, we found that precipitate slowly forms on prolonged standing at this higher temperature. Proton spectra recorded in these temperature ranges are virtually identical. ^{15}N chemical shifts were referenced to 99% ^{15}N-enriched *N*-acetylglycine (90.4 ppm) as external standard. ^{15}N NOE suppressed and $^{15}N\{^1H\}$ NOE spectra were collected over a sweep width of 10 kHz, with 72° pulses and a relaxation delay of 8 s. A total of 9 W of broad-band proton composite decoupling was applied during acquisition, and in the case of the NOE spectra, 0.4 W of continuous broad-band proton decoupling was applied during the relaxation delay. A total of 4K points was collected, which was zero-filled to 8K prior to Fourier transformation; typically between 7000 and 20 000 scans were collected to give acceptable signal to noise. DEPT spectra were typically collected over 10 kHz, with either a final proton pulse of 135° to discriminate between NH and NH_2 groups or a 90° pulse to selectively observe NH groups, as appropriate. DEPT spectra were optimized for a 95-Hz $^1J_{NH}$ coupling. A total of 9 W of broad-band proton composite decoupling was applied during acquisition; 4K points were collected, which were zero-filled to 8K prior to Fourier transformation; typically between 1000 and 4000 scans were collected for adequate signal to noise for uniformly labeled *cro* repressor and 15 000–40 000 scans for single amino acid labeled samples. Free induction decays were apodized with 3–10 Hz of exponential line broadening. High signal to noise DEPT spectra were given a mild Gaussian filter to increase resolution.

Two-dimensional proton-detected multiple-quantum ^{15}N–^1H correlation spectra (Müller, 1979; Griffey et al., 1983, 1985; Bax et al., 1983; Live et al., 1984) were recorded with phase-sensitive detection by the method of States et al. (1982). The preparation period was 5.28 ms ($1/2J_{NH}$). The water resonance was presaturated during the 2-s recycle delay. A total of 9 W of composite ^{15}N decoupling was applied during acquisition, so that the resultant spectrum was heteronuclear decoupled in both dimensions. A total of 4K points were collected over 4.5 kHz in t_2, and 64 time domain points were collected over 1.2 kHz in t_1, with 1024 scans being collected at each t_1 point. The matrix was apodized, with 5 Hz of exponential line broadening in t_2 and a 90° phase-shifted sine bell in t_1, zero filled in t_1, and transformed to a final size of 2K × 128 points. Proton chemical shifts were referenced to internal TSP. Processing was performed on a Microvax II with software supplied by Dennis Hare of Infinity Designs Inc.

DNA Titrations. The titrations of labeled *cro* repressor with O_R3 were performed by the addition of aliquots of a concentrated O_R3 solution to a final concentration of 1.1 duplexes to 1 *cro* dimer. On completion of the titration, reversibility was demonstrated by the addition of aliquots of 4 M KCl to a final concentration of 0.77 M KCl. Similarly for the nonspecific DNA titrations, aliquots of a 22.5 mg/mL solution of DNA fragments were added to a solution of *cro* repressor to a final concentration of either 40 or 80 base pairs per *cro* dimer. The complexes were dialyzed against either phosphate or citrate buffer so that the effect of changing pH could be monitored. Finally, the complexes were returned to the original buffer; reproducible spectra indicated that no degradation of either protein or DNA had occurred.

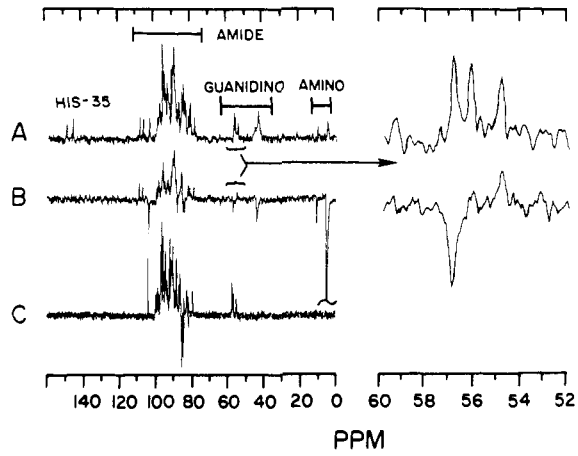

FIGURE 2: ^{15}N NMR spectra of uniformly labeled *cro* repressor, pH 4.6, in 10 mM citrate, 2 mM EDTA, and 0.2 M KCl, 22 °C. (A) NOE-suppressed spectrum, 8000 scans. (B) NOE spectrum, 8000 scans. (C) DEPT spectrum recorded with a 135° final proton pulse, 1000 scans. Expansion of arginine NH guanidino resonances (right): NOE suppressed (upper) and NOE (lower).

Carboxypeptidase Digestion. For identification of the C-terminal alanine, uniformly ^{15}N-labeled *cro* repressor was digested with carboxypeptidase A (PMSF treated, Sigma) 0.01 equiv, 0.2 M Tris-HCl, pH 8.5 (Ambler, 1967). Lysines-63 and -62 were identified by digestion of [α-^{15}N]Lys-labeled *cro* repressor with both carboxypeptidases A and B (DFP treated, Sigma), 0.01 equiv of each, 0.2 M Tris-HCl, pH 7.3, for 1 h at 25 °C. After digestion the pH was lowered to 5.0 to reduce the enzyme activity and so that all amides were detectable by DEPT.

RESULTS

^{15}N Spectroscopy of cro Repressor. (1) General Aspects. Spectra were recorded at various pHs in order to investigate the effect of pH on the rate of proton exchange for the various nitrogens in *cro* repressor. The imidazole group of the sole histidine, His-35, is titrated as the pH is lowered below 6, and accordingly, these nitrogens undergo large chemical shift changes (Smith et al., 1987). As the pH is lowered below 4.5, the carboxylate side chains of Glu and Asp and the C-terminus are titrated. Many chemical shift changes are then evident in the amide region. The DEPT, NOE, and NOE-suppressed spectra of *cro* repressor at pH 4.6 are shown in Figure 2. The DEPT spectrum (Figure 2C) shows positive peaks due to either NH or NH_3 groups and negative peaks due to NH_2 groups; thus, the negative peaks at ~85 ppm are amide NH_2 resonances due to glutamine and asparagine. At this pH only five peaks are resolved for the six side-chain amides; however, on raising the pH to 65, all six NH_2 resonances are resolved.

It is clear that the DEPT spectrum in Figure 2C contains only a subset of the resonances detected by the other two techniques. Specifically only amides and the guanidino δ-nitrogens are present in the spectrum. This effect could be either due to the other nitrogens having vastly different $^1J_{NH}$'s or due to fast proton exchange, with the solvent, on the time

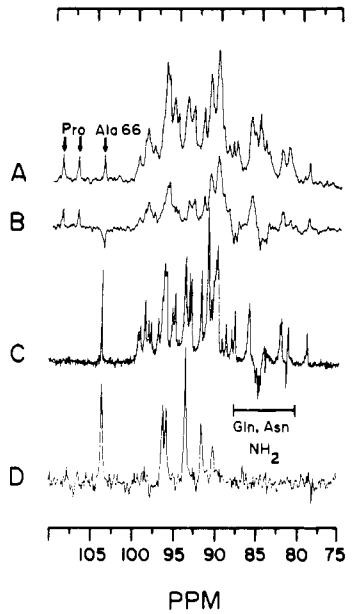

FIGURE 3: Expansions of the amide region of uniformly (A–C) and specifically (D) ^{15}N-labeled *cro* repressor spectra: (A) NOE suppressed; (B) NOE; (C) DEPT; (D) DEPT of [^{15}N]Ala-labeled repressor.

scale of $1/J_{NH}$ (10 ms). A series of DEPT spectra optimized throughout the known range of typical ^{15}N–^{1}H coupling constants (60–75 Hz for sp^3-hybridized nitrogen and 90–100 Hz for sp^2) does not produce the missing resonances, and on raising the pH to 7, the guanidino δ-nitrogen signals weaken and finally disappear. Yavari and Roberts (1978) have studied the ^{15}N NMR spectrum of arginine over the pH range 0.5–11.5. They report that the $^{1}J_{NH}$ values for the two nitrogens are virtually identical (δ-N, J = 91 Hz; ω-N, J = 92 Hz) and find, from line-shape analysis, that the δ-N has a faster proton exchange rate than the ω-N. It is clear from our data that this is not the case.

It seems, therefore, that although DEPT provides a useful gain in sensitivity (γ_H/γ_N), signals are lost from the nitrogens with more rapidly exchanging protons, which at neutral pH causes the loss of a large number of resonances.

Spectra A and B of Figure 2 show the NOE-suppressed and NOE spectra, respectively. The presence of large negative peaks in the latter shows the presence of nitrogen sites that are rapidly reorienting on the time scale of the Larmor frequency (Figure 1), e.g., the amino resonances. Several negative peaks are visible in the amide region (Figures 2 and 3), which do not arise from the side-chain amides of either Gln or Asn. This therefore indicates the presence of one or more mobile domains in the protein backbone. The amino resonances appear to be attenuated in the NOE-suppressed spectrum, since they exhibit a larger than theoretical NOE. These effects are due to an insufficiently long relaxation delay (8 s) and will be most marked at the more mobile sites.

The right-hand portion of Figure 2 illustrates the ability of the heteronuclear ^{15}N{^{1}H} NOE to resolve differential dynamics within the protein. The expansion of the region of the spectrum showing the guanidino δ-nitrogens shows three peaks due to Arg-4, -13, and -38; one of these exhibits a ca. −200% NOE, one a −100% NOE, and a small negative NOE. In the crystal structure of *cro* repressor the guanidino group of Arg-4 is hydrogen bonded to the carboxylate of Glu-2 and sits in a cleft at the dimer junction, whereas the other two arginine guanidino groups are at the surface exposed to solvent (Anderson et al., 1981). This experiment therefore suggests that the positive resonance is due to Arg-4; proof of this would require removal of this residue by site-directed mutagenesis.

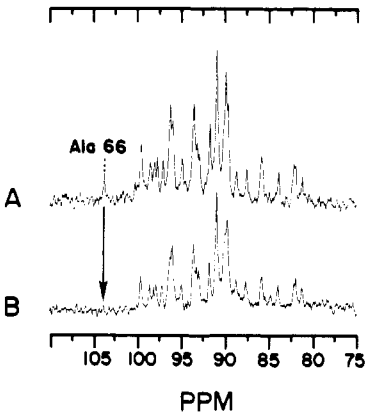

FIGURE 4: DEPT spectra, recorded with a 90° final proton pulse, of uniformly ^{15}N-labeled *cro* repressor in 0.2 M Tris-HCl, pH 8.5, before (A) and immediately after (B) the addition of 0.01 equiv of carboxypeptidase A. A total of 1000 scans were recorded in each case with a 1.5-s recycle delay.

(*2*) *Assignment of C-Terminal Residues.* In the expansion of the amide region of these spectra (Figure 3) two resonances at low field that are present in the NOE and NOE-suppressed spectra are absent from the DEPT spectrum. These we assign to the two prolines, 57 and 59, since these residues lack an amide proton and are accordingly not detectable by DEPT.

It has been suggested that Pro-59 is in slow exchange on the proton chemical shift time scale ($\sim 10^{-3}$ s) between cis and trans isomers (Weber et al., 1985). This suggestion was based on the apparent nonobservation of cross-peaks for Pro-59 and the splitting of the resonances of Ser-60, Asn-61, and Phe-58 in the absolute value COSY spectrum [the assignment of Phe-58 has recently been questioned (Anderson et al., 1986)]. We therefore expect to observe two resonances for Pro-59; however, we in fact observe two singlets of equal intensity (Figure 3).

The dipeptide alanylproline, which from studies of both ^{13}C (Evans & Rabenstein, 1974; Gierasch et al., 1982) and ^{15}N (Blomberg et al., 1978) spectra, has been reported to undergo slow cis–trans isomerism, being approximately 35% as the cis isomer. ^{15}N NMR spectra of the zwitterion of Ala-[^{15}N]Pro showed two lines separated by 0.8 ppm (32 Hz at 9.4 T) at 30 °C, a similar result to that obtained for *N*-formyl- and *N*-acetylproline (Hawkes et al., 1975). Since proline isomerism is generally a slow phenomenon ($k \simeq 10^{-1}$–10^{-2} s^{-1}, $E_{act} \simeq$ 80 kJ mol^{-1}) (Brandts et al., 1975) and the time scales of proton and nitrogen chemical shifts are not vastly different, given the large nitrogen chemical shift dispersion, it seems that the previously conjectured exchange does not occur. This means that the C-terminal arm *does not* sweep through large regions of space as the proline peptide bond isomerizes but rather is held static on its N-terminal side.

The C-terminal residue of *cro* is an alanine, Ala-66. In order to assign this residue, we selectively labeled the protein with [^{15}N]alanine. The DEPT spectrum in Figure 3 shows the resonances of the eight alanine amides of *cro*. Inspection of Figure 3 shows that the well-resolved resonance in the uniformly labeled spectrum at 103.7 ppm is therefore an alanine amide. Furthermore, the NOE spectrum indicates that this is a mobile residue and, hence, likely to be Ala-66. To establish this, we digested a sample of the uniformly labeled protein with carboxypeptidase A, monitoring the progress of the digestion by DEPT. The liberated amino acids will have amino nitrogens, which due to their fast proton exchange rate are not detectable by this technique (Figure 4). The signal due to the mobile alanine rapidly vanishes while almost all other

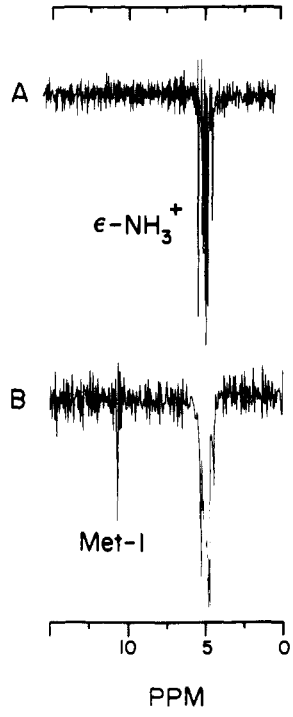

FIGURE 5: Amino region of the NOE spectra of (A) [α,ϵ-$^{15}N_2$]-Lys-labeled *cro* repressor and (B) uniformly labeled *cro* repressor, pH 4.6, in 0.2 M KCl, 10 mM citrate, and 2 mM EDTA.

Table I: Proton and Nitrogen Chemical Shifts of *cro* Lysine Amides and Changes in Nitrogen Chemical Shift on Addition of Operator DNA

$\delta_{^{15}N}$[a]	$\delta_{^1H}$[a]	$\Delta\delta_{^1H}$[b]	assignment[c]	$\Delta\delta_{^{15}N}$ (complex − free)[d]
90.7	7.81	+0.26		−0.09
89.7	8.23	−0.15		0.43
93.6	8.27	−0.02	62	nd[e]
95.0	8.48	−0.19	63	0.37
88.4	8.56	+0.18		0.03
95.7	8.91	+0.02		0.06
98.2	9.21	−0.16		0.14
99.1	9.35	−0.22	56	0.70

[a] Chemical shift of lysine amides of labeled *cro*, pH 4.6, 0.2 M KCl, 23 °C. Proton chemical shifts are listed in ascending order relative to internal TSP. Nitrogen chemical shifts are relative to *N*-acetylglycine at pH 4.5 (90.4 ppm). [b] Differences in chemical shift (in ppm) for the eight lysine amide protons, listed in ascending order of chemical shift, relative to the values reported by Weber et al. (1985). [c] This work. [d] Differences in chemical shift (in ppm) for the lysine amide nitrogen resonances in O_R3–*cro* complex (1.1:1) and free *cro* repressor (conditions: pH 6.8, 0.2 M KCl, 10 mM P_i, 2 mM EDTA, 26 °C). [e] Not determined (signal is not detected in free *cro* at this pH).

signals remain with full intensity, and we assign this resonance to Ala-66. Carboxypeptidase A will digest, at most, the seven C-terminal residues, since it is unable to digest the Phe-58–Pro-59 bond. Of the seven C-terminal residues only 66 is an alanine. The poorer signal to noise in the DEPT spectra in Figure 4 compared to that in Figure 3 is due to the higher pH (8.5) causing more rapid proton exchange and consequent loss of the DEPT signal.

Having unambiguously assigned the C-terminal alanine, we next sought the assignment of other residues in the C-terminus. Residues 65 and 64 are both threonines, and 63 and 62 are both lysines. [^{15}N]Threonine scrambles over many sites (Schneider, 1987); however, [α-^{15}N]lysine has been found to give no detectable scrambling of the label and to give a high level of incorporation (M. Bogusky, personal communication). We therefore decided not to label the threonines but to specifically label the eight lysines in *cro* repressor. By labeling

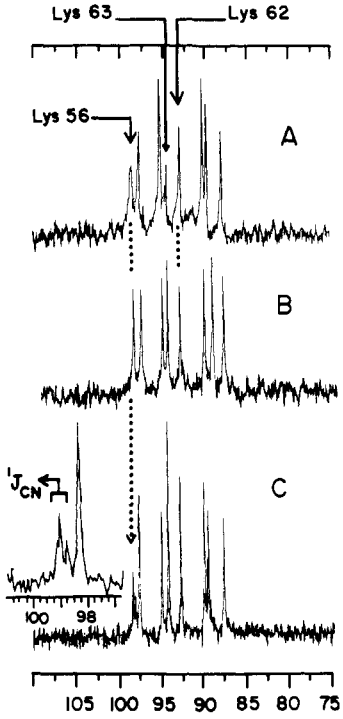

FIGURE 6: DEPT spectra of [α-^{15}N]Lys-labeled *cro* repressor. (A) After digestion with carboxypeptidases A and B, pH 5.0, 0.2 M Tris-HCl. Assignments of Lys-62 and -63 are shown (B) before enzymatic digestion, pH 4.6, 0.2 M KCl, 10 mM citrate, and 2 mM EDTA. (C) [α-^{15}N]Lys,[1-^{13}C]Val double-labeled *cro* repressor, pH 4.6, 0.2 M KCl, 10 mM citrate, and 2 mM EDTA. Assignment of Lys-56 is shown. (D) Expansion of (C) showing Lys-56 resonance.

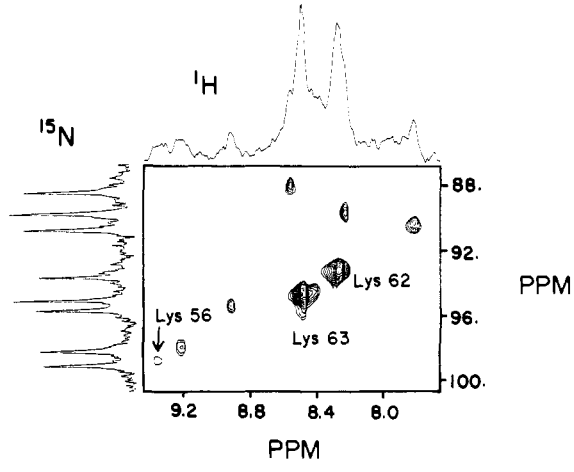

FIGURE 7: Pure absorption two-dimensional multiple-quantum heteronuclear ^{15}N–^1H chemical shift correlation spectrum of [α,ϵ-$^{15}N_2$]Lys-labeled *cro* repressor obtained as described in the text. The DEPT spectrum is displayed along the nitrogen dimension, and the transform of the first row of the data matrix is shown along the proton dimension.

both the α- and ϵ-nitrogens in the lysine, we would also be able to account for all of the amino groups except the N-terminus of the protein, and thus also obtain the assignment of the N-terminal residue. Figure 5 shows the amino regions of the NOE spectra of uniformly labeled and [α,ϵ-$^{15}N_2$]Lys-labeled *cro* repressor; the additional resonance at 11 ppm in the spectrum of the uniformly labeled protein is due to Met-1. The DEPT spectrum of the amide region of [α,ϵ-$^{15}N_2$]Lys-labeled *cro* repressor shows eight well-resolved resonances due to the eight lysines present in the protein (Figure 6). In order to establish the identities of these resonances, we sought to take advantage of the previously reported proton assignments of

cro repressor (Weber et al., 1985) and use heteronuclear chemical shift correlation. An examination of the heteronuclear chemical shift correlation, Figure 7, and Table I indicates that our measured proton chemical shifts are inconsistent with those reported by Weber et al. (1985). We discuss this further below.

In order, therefore, to assign the amides at Lys-62 and -63 of the C-terminal arm, we again digested the protein with carboxypeptidases. However, digestion of *cro* repressor with both carboxypeptidases A and B (the latter present to remove lysine more efficiently) produces an insoluble product. Hence, in order to assign the amide resonances of the lysines, we did a partial digestion that would remove most of Lys-63 and a fraction of Lys-62. A number of attempts to remove both lysines with these enzymes showed that precipitation was caused by their removal. Figure 6B shows the amide region of the DEPT spectrum obtained by digesting *cro* repressor with 0.01 equiv of both carboxypeptidases for 1 h at pH 7.3. The spectrum is recorded at pH 5.0. The resonance at 95.0 ppm is now absent, and the resonance at 93.6 ppm is considerably attenuated with respect to Figure 6A. Hence, we assign the former to Lys-63 and the latter to Lys-62. Broadening is apparent in the resonance at 99 ppm. We show below that this resonance is that of Lys-56, and hence, the broadening is due to lysine-56 existing in several different environments, due to species in various states of C-terminal digestion. Lysine-56 as the nearest lysine is most greatly affected by this.

It is possible to assign the remaining six lysine amide resonances by double labeling the protein with both ^{13}C and ^{15}N, a strategy recently used by Griffey et al. (1986). We illustrate this by the multiple labeling of *cro* with both [α-^{15}N]Lys and [1-^{13}C]Val, which uniquely colabels the peptide bond of Lys-56–Val-54. The resonance of Lys-56 is then readily identified in the DEPT spectrum due to the ^{13}C–^{15}N scalar coupling of approximately 15 Hz (Kainosho & Tsuji, 1982). This is shown in Figure 6C. The Lys-56 resonance at 99 ppm is composed of a doublet (J = 13.6 Hz) due to the nitrogens adjacent to ^{13}C and a singlet due to the nitrogens adjacent to ^{12}C. This latter peak arises due to the incomplete labeling of the valine carbonyls (we estimate an approximately 60% incorporation of label). There is clearly a significant isotope effect on the nitrogen due to the ^{13}C. The quality of the spectrum precludes an accurate measurement of the isotope shift: the value we measure is $^1\Delta$ = 35 ± 15 ppb (upfield for ^{13}C).

In contrast to the two-dimensional proton-detected method suggested by Griffey et al. (1986) for detecting ^{13}C scalar coupled nitrogens, which gives poor resolution due to short proton t_2's, the DEPT method clearly provides considerably higher resolution and thus is applicable to both higher molecular weight systems and to cases of low ^{13}C incorporation. In conjunction with a single two-dimensional ^{1}H–^{15}N map, proton chemical shifts may be correlated with each doubly labeled site.

NOE spectra of [^{15}N]Lys-labeled *cro* (Figure 8) show that two of the eight lysines, corresponding to residues 62 and 63, have enhanced mobility with respect to the other six. Moreover, this mobility is partially pH dependent. At pH 4.6 six peaks are positive and two are negative with approximately the same intensities, whereas at higher pH (pH 6.5) Lys-63 is more intensely negative while Lys-62 is almost nulled. Thus residues 66–62 (and possibly more) form a mobile domain in solution, and in neutral solution we observe a gradient of mobility, with motion increasing toward the C-terminal end of the arm.

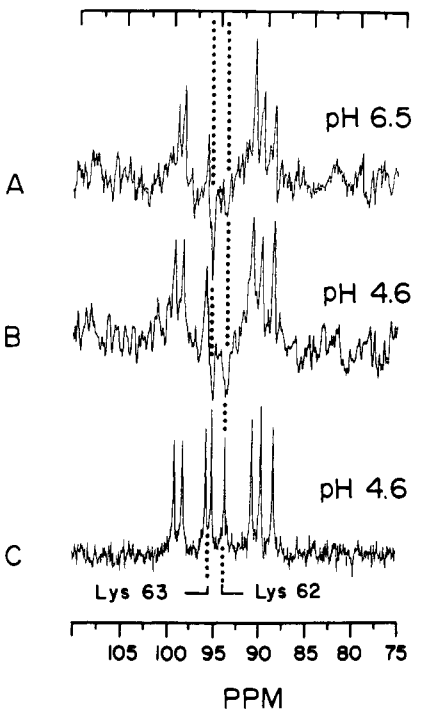

FIGURE 8: (A) Amide region of the NOE spectrum of [α,ϵ-^{15}N$_2$]-Lys-labeled *cro* repressor, 20 000 scans, pH 6.5. (B) Amide region of the NOE spectrum of [α,ϵ-^{15}N$_2$]Lys-labeled *cro* repressor, 20 000 scans, pH 4.6. (C) Amide region of the DEPT spectrum recorded with a 90° final proton pulse of [α,ϵ-^{15}N$_2$]Lys-labeled *cro* repressor, pH 4.6, 17 000 scans.

On closer inspection of Figure 7, it is apparent that there are two sets of cross-peaks: two strong resonances and six weaker ones. The strong resonances correlate with the two lysines that exhibit the largest NOE and are hence the most mobile. Clearly we are observing a dynamic filtration in this experiment due to the shorter proton t_2's of the less mobile nitrogens. Thus we are observing considerably less than the theoretical maximum $(\gamma_H/\gamma_N)^{5/2}$ sensitivity enhancement potentially offered by this technique, which is evident in the case of the more rigid lysines. We did not optimize this experiment for proton t_2 decay, using $1/2J$ preparation and refocusing periods, which generate maximum multiple quantum coherence. Optimization of these delays for coherence transfer vs t_2 decay will partially offset the signal losses due to proton t_2 decay, at the cost of less coherence transfer. Studies of large macromolecules are limited by this problem.

The relative exchange rates of the eight lysine amides may measured by DEPT as a function of pH. A series of DEPT spectra taken at differing pHs (Figure 9) shows that although all resonances are visible at pH 5.5, as the pH is raised the amide protons of Lys-62 and Lys-63 exchange most rapidly, being barely detectable at pH 6.8 though the other resonances are present with almost full intensity. As the pH is raised further other resonances disappear. Finally at pH 10.5, still below the alkaline denaturation point of *cro*, only two signals remain. The stronger of these at 99 ppm is the due to Lys-56 (see above), and thus this is the most slowly exchanging lysine amide in the protein. The rapid amide proton exchange rates of Lys-62 and Lys-63 are therefore consistent with the NOE results.

In 0.2 M Tris-HCl buffer, 26 °C, Lys-62 and -63 have ~45% intensity at pH 6.4, as at pH 4.6 (calculated with respect to the most slowly exchanging amide, that of Lys-56). The random-coil polypeptide poly(DL-alanine) has an exchange half-life of ~30 ms under these conditions (Englander & Poulsen, 1969) for tritium–proton exchange. The kinetic

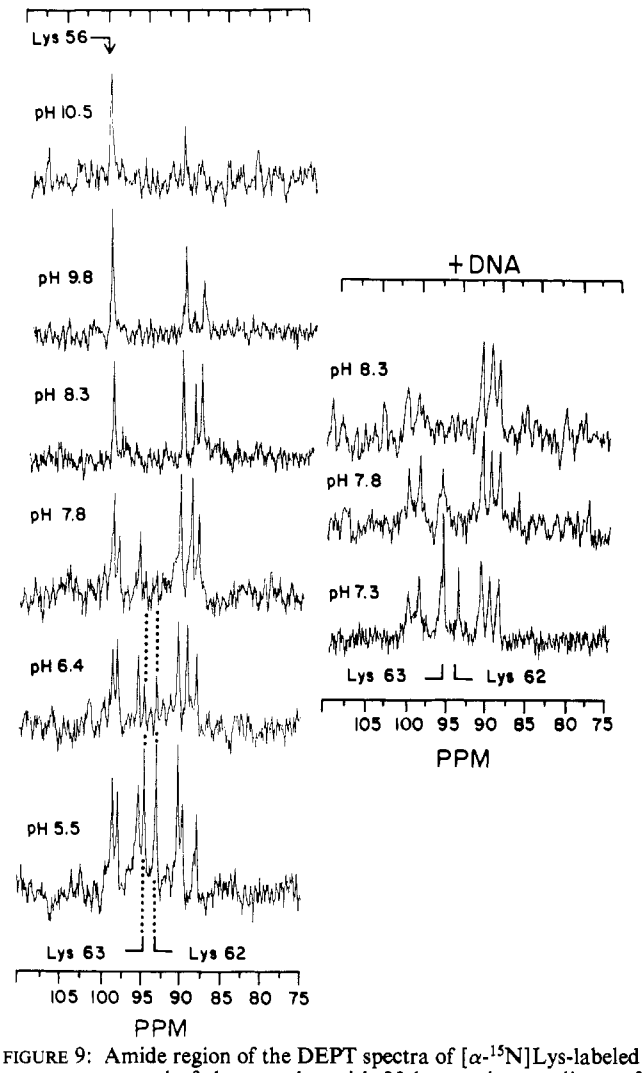

FIGURE 9: Amide region of the DEPT spectra of [α-^{15}N]Lys-labeled *cro* repressor and of the complex with 80 base pairs per dimer of nonspecific DNA, in 0.2 M Tris-HCl at varying pHs.

isotope effect for tritium causes probably no more than a factor of 2 in the exchange rate (S. W. Englander, personal communication). Correcting the above value for the appropriate Molday factors (Molday et al., 1972) gives exchange half-lives for the two lysines of ~11 ms. Increasing ionic strength causes a decrease in amide exchange of poly(DL-lysine) but not poly(DL-alanine) (Kim & Baldwin, 1982). These charge effects are small except in regions of high local charge density. Since both our measurements and those of Molday et al. (1972) were made at identical ionic strengths (0.2 M), any correction for such effects will be insignificant. In the DEPT experiment the time delay between the first proton pulse and the transfer of proton polarization to ^{15}N is 10.6 ms ($1/J_{NH}$). The observed half-life of the amide protons of Lys-62 and -63 are thus, within the approximations of this calculation, identical with those expected for a random-coil peptide. In summary, the C-terminal arm is fully solvent accessible and is undergoing rapid motions similar to a random-coil peptide.

The amide proton exchange rates of Lys-62 and -63 are considerably faster than that of Ala-66 (compare Figures 4 and 9), whose amide is still observable at pH 8.5. This is primarily due to the inductive effect of the negatively charged carbonylate group, which reduces the base-catalyzed exchange rate of the C-terminus, and also to the intrinsically lower exchange rate of alanine (Molday et al., 1972).

^{15}N Spectroscopy of cro–DNA Complexes. (1) General Observations. No large chemical shift changes may be discerned during the titration of uniformly ^{15}N-labeled *cro* with either O_R3 operator or nonspecific DNA, by either DEPT or NOE-suppressed techniques. Any smaller changes that occur in the amide region are obscured by the considerable number of overlapping resonances. Some subtle changes are apparent in the lysine amino resonances, but these are smaller than those induced by a change in the ionic strength from 50 to 200 mM KCl and probably reflect the changes in ionic environment as these groups interact with the DNA phosphates. The lack of any major changes in the ^{15}N chemical shifts of the free and bound protein indicates that there are no structural changes in the protein on complexation detectable by ^{15}N NMR.

The changes that do occur in the amide region are more readily resolved by individual amino acid labeling. The titration of [^{15}N]Lys-labeled *cro* with O_R3 (Table I) reveals that the amide resonances of some lysines (specifically 56, 63, and one other as yet unassigned resonance) are significantly shifted, whereas other resonances are almost unchanged. The chemical shift changes, 0.7 and 0.37 ppm, observed for Lys-56 and -63, respectively, are consistent with the model of Ohlendorf et al. (1982), which has the amino groups of these residues interacting with the phosphate backbone of the DNA. Other lysines, 39, 21, 18, and 8, are more distant from the binding site and interact weakly or not at all, and hence if the model is correct, we would predict that these resonances are the four least-shifted ones.

DEPT spectra of the complex of uniformly ^{15}N-labeled *cro* with both O_R3 and nonspecific DNA show that there is a reduction in the signal intensity upon complexation. This results from the changed hydrodynamic properties of the protein, which is now tumbling more slowly, due to complex formation, and thus has reduced proton t_2's, giving less efficient polarization transfer. The reduction in t_2 is more pronounced on the higher gyromagnetic nucleus (^{1}H), which explains why no significant broadening is observed in the NOE-suppressed spectra. Furthermore, the DEPT spectra show the presence of some shifts and broadening of certain of the NH_2 groups of Gln and Asn. The model of Ohlendorf et al. (1982) for *cro*–O_R3 interaction predicts that four of the six amide side chains are involved in hydrogen bonding, one to an adenine base and three to the phosphate backbone. The changes we see in the DEPT spectra are consistent with these interactions.

NOE spectra of the complexes formed with both specific operator and nonspecific DNA show that there are no detectable changes in the dynamics of the side chains of either Arg, Lys, or His. However, changes are observable in the amide region (Figure 10).

(2) The C-Terminus. The Ala-66 resonance, which is inverted in the NOE spectrum of the free protein, is nulled when a 1:1.1 (protein dimer: O_R3 DNA 17-mer) complex with specific operator at pH 6.5 is formed or when 40 base pairs of nonspecific DNA per dimer are added (approximately two binding sites per dimer) (Figure 10). This result indicates that the mobility of the C-terminal residue is reduced in the presence of the DNA, and it is apparent that this interaction occurs with both specific operator DNA and with random fragments. However, as Figure 10A shows at pH 4.6 the mobility of the alanine is unaffected by binding to DNA. This is true for both the specific and the nonspecific complex (data not shown). It is conceivable that the protein does not bind at this reduced pH; however, several observations contradict this hypothesis: first, a reduced DEPT signal is obtained, indicative of short proton t_2's, and second, the rotational correlation time of ethidium-labeled DNA fragments is increased by *cro* repressor at this pH (Cook and Lu, personal

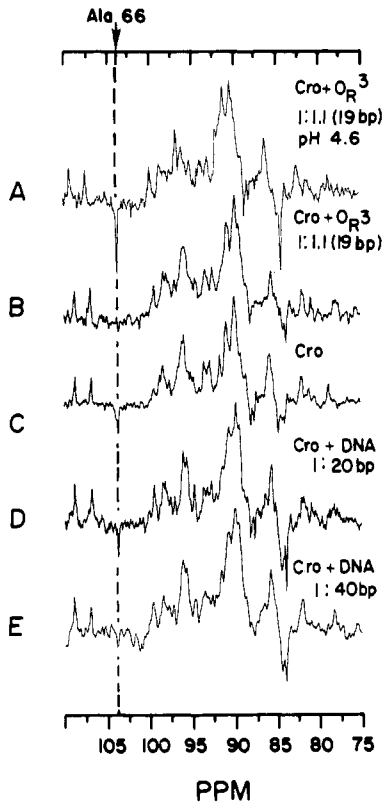

FIGURE 10: NOE spectra of the amide region of uniformly ^{15}N-labeled *cro* repressor: (A) *cro*–O_R3 complex (pH 4.6); (B) *cro*–O_R3 complex (pH 6.5); (C) *cro* repressor only (pH 6.5); (D) *cro* repressor + 20 bp/dimer of nonspecific DNA (pH 6.5); (E) *cro* repressor + 40 bp/dimer of nonspecific DNA (pH 6.5).

communication). We therefore explain this result as being due to either an altered mode of binding at low pH that does not involve the C-terminal arm or the carboxyl terminus participating in the binding of the DNA, perhaps by hydrogen bonding to a guanosine NH_2 in the minor groove. In the latter case, lowering the pH to near the pK_a of the carboxylic acid may reduce the binding and produce the observed enhancement in mobility.

We have therefore shown that complexes formed between *cro* repressor and both specific and nonspecific fragments have many similarities under these conditions; it is thus possible to use relatively less expensive nonspecific DNA fragments for many exploratory experiments.

Other changes are also apparent in the NOE spectrum of the complex; there is more positive intensity present in the spectrum of the bound than of the free protein, due to all nitrogens receiving a smaller net negative NOE since the rotational correlation time has now increased (Figure 1); also, two negative peaks are present at 88–89 ppm in the free protein, whereas in the specific complex one of these is nulled, possibly indicating a change in dynamics of another residue. The degree of overlap for uniformly ^{15}N-labeled *cro* repressor is too great for the majority of amides to clearly resolve subtle changes in dynamics for more than a few residues; this problem may be surmounted either by a two-dimensional heteronuclear NOE correlation experiment (Yu & Levy, 1984) or by the use of single amino acid labels.

NOE spectra of the complex formed between nonspecific DNA and [^{15}N]Lys-labeled *cro* repressor show no detectable change at either 40 or 80 base pairs per dimer at pH 6.5. The poor signal to noise in these spectra may mask some subtle changes in NOE. However, it is clear that in the nonspecific complex Ala-66 exhibits a ca. −100% NOE and is hence nulled, whereas the amide nitrogen of Lys-63 exhibits a measurably greater NOE. The reason for the greater mobility at the backbone of Lys-63 is not at present clear. The interaction between the two C-terminal lysines and nonspecific DNA fragments is revealed, however, by observing DEPT spectra at different pHs. Whereas the resonances for the amides of Lys-62 and -63 in the free protein are not observable above pH 7 and are considerably weakened above pH 6, in the complex these two resonances are still intense at pH 7.3 (Figure 9), indicating that binding to DNA protects these amides from exchange with the solvent. Since we have shown above that these residues form part of an extended, mobile C-terminal arm in the free protein, clearly in the complex this arm must be partially protected from exposure to the solvent. This is most logically performed by binding of the arm within one of the grooves of the DNA.

On increasing the pH of the complex, the DEPT signal rapidly decays; at pH 7.8 the resonances of Lys-62 and -63 are no longer detectable, and at pH 8.5 no signal is detectable even after 2 days of signal averaging. This rapid loss of DEPT signal with increasing pH is due to both proton exchange and the short proton t_2's of the complex contributing to the reduced polarization transfer. Due to the differing proton t_2's, it is not possible to quantitatively compare DEPT intensity in the free protein and in the complex. Whereas in the free protein the amide of Lys-56 is significantly more slowly exchanging than those of the other lysines, this does not appear to be the case in the complex (Figure 9). Small distortions of the β-sheet joining the two monomers together caused by movement of the subunits on binding could perhaps cause this change in exchange rate.

cro–O_R3 complex freshly dissolved in D_2O at pHs between 3 and 7 did not show any amide resonances by DEPT. Thus all amide protons are in exchange with the solvent on a minute time scale. This behavior has been noted previously for the free protein (Weber et al., 1985) and is highly unusual for a globular protein; binding to DNA apparently does not substantially affect the stability of the secondary structure of the protein.

Discussion

Although offering exceptional resolution, the ^{15}N NMR spectrum is not easy to assign. Sequential nitrogens within the backbone are too distantly connected to display either spin–spin coupling (three bonds) or cross-relaxation, since nitrogen relaxation is dominated by dipolar relaxation to the attached proton or by chemical shift anisotropy if the nitrogen is unprotonated. The only possible ways of assigning the spectrum are through ^{15}N–^{1}H correlation, if the proton chemical shifts are known, or by selective labeling. The former has disadvantages in practice since the prime utility of nitrogen NMR would be in cases where the proton spectrum is intractable. In addition, correlation is only possible if the integrity of the proton–nitrogen bond is maintained throughout the pulse sequence, which, as we saw above in the DEPT spectra, is not always the case. The advantages of selective labeling is that no prior knowledge of the proton spectrum is required, and in the absence of scrambling, all sites are accessible to this technique. Since numerous amino acids of any given type are normally present, individual residues may be assigned either by site-directed mutagenesis (followed by the corresponding ^{15}N NMR observations) or by the double labeling of unique C–N pairs with a [^{13}C]carbonyl-labeled amino acid that is adjacent to the desired ^{15}N site.

The proton assignments of the backbone and most side-chain resonances of *cro* repressor have been reported previously

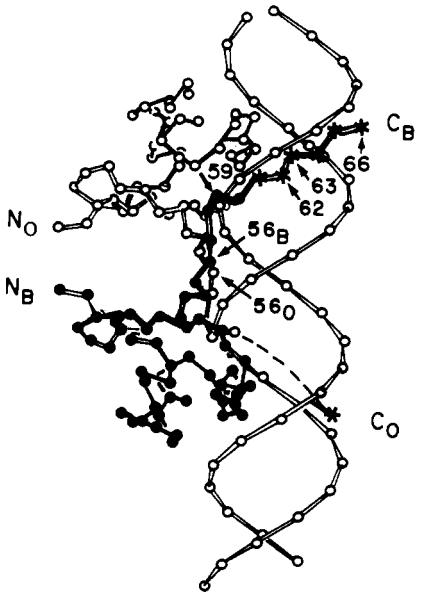

FIGURE 11: Representation of the *cro*–O_R3 complex based on the model of Ohlendorf et al. (1982) showing the C-terminal arm of the repressor lying in the minor groove of B-form DNA. The α-carbons of residues 1–60 (filled circles for subunit B and open circles for subunit O) were located as in the crystal structure of the protein (Anderson et al., 1981), and the phosphate backbone of the DNA is shown on the basis of the model. The remaining residues, indicated with an asterisk (*) (61–66) have been arranged in a suitable geometry to allow interaction with the phosphates by Lys-62 and -63. The α-carbons of Pro-59 and Lys-62 and Lys-63 are indicated with arrows. The α-carbons of the two lysine-56's are indicated by 56_B and 56_O—note their close proximity in the structure. The C-terminal arm of subunit O passes behind the helix and is indicated with a dotted line. A space-filling version, in color, of this schematic diagram may be found on the cover of Watson et al. (1987).

(Weber et al., 1985), and it would have been straightforward to assign the nitrogen resonances by heteronuclear correlation. However, inconsistencies in the reported chemical shifts of amide protons and those that we measured for a given amino acid type (Table I, column 3), in this case lysine, suggest that there are errors in the sequential assignment of Weber et al. (1985). If the previous assignments were correct, then we would expect a constant difference in column 3 of Table I. The two sets of spectra, those of Weber et al. (1985) and those we show here, have been acquired at identical pHs and ionic strength but slightly different temperatures. One-dimensional spectra of *cro* repressor do not show significant chemical shift changes within this temperature range. We note that the eight alanine amide resonances of [^{15}N]Ala-labeled *cro* agree with the published values of Weber et al. (1985), suggesting that the differences in lysine amide shifts are not due to differences in sample preparation or conditions.

We have therefore assigned those ^{15}N resonances of C-terminal residues as were necessary by isotopic labeling with single amino acid labels, combined with either enzymatic digestion or ^{13}C–^{15}N multiple labeling, to obtain residue specific assignments. We note here the value of the proton-detected multiple-quantum heteronuclear correlation experiment of single amino acid type labels as an independent check of amide assignments made by homonuclear proton spectroscopy.

Clearly from the NOE spectra of *cro* repressor (Figures 2 and 3), it is tumbling in solution as a globular protein, with all amides exhibiting an approximately equal NOE, with the exception of a few considerably more mobile sites. A closer examination has revealed that these mobile residues are situated in the C-terminus of the protein. This mobile domain was invisible to X-ray crystallography. We have shown that the C-terminus is partially immobilized in the presence of stoichiometric amounts of specific operator, and larger amounts of nonspecific DNA, and that amide proton exchange within the C-terminal arm is slowed in the presence of DNA. We have further shown that a purported proline isomerization is not occurring. These findings reinforce the earlier suggestions about the basis of *cro*–O_R3 interaction at a molecular level, and this is illustrated in Figure 11. These data represent direct spectroscopic observation of C-terminal involvement in DNA binding, as previously implied by chemical modification of the lysines at residues 62 and 63 (Takeda et al., 1986) and by mutagenesis (Caruthers et al., 1986; Gayle et al., 1986). The C-terminus of *cro* repressor is thus structurally and functionally analogous to the N-terminal arm of λ phage c1 repressor (Pabo et al., 1982).

We have demonstrated that ^{15}N NMR spectroscopy is a useful technique for the study of mobile domains in proteins. This is due to the large change in the heteronuclear NOE on the nanosecond time scale, which corresponds to the time scale for segmental motion in proteins (Jardetzky & Roberts, 1981). This technique should be particularly useful since such sites may be of interest in the binding of substrates and cofactors. We have also used ^{15}N NMR in a novel way to examine proton exchange rates and have shown that, even using a crude qualitative approach, useful information about a relatively large complex of interacting macromolecules is readily extracted.

ACKNOWLEDGMENTS

We are grateful to Dr. S. J. Opella, Michael Bogusky, and Robert Schiksnis for their advice and encouragement and also to Phoebe Stewart for the gift of [α,ϵ-$^{15}N_2$]-L-lysine. O_R3 DNA was synthesized by the University of Pennsylvania Cancer Center DNA synthesis service by Dr. M. Mitchell.

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