tography using the same conditions described above for the analysis of styrene oxygenation products. Under these conditions, the observed retention times were as follows: *N,N*-dimethylaniline, 9.3 min; *N*-methylaniline, 10.3 min; and ethyl benzoate, 14.7 min.

Preparation of a 5′-^{32}P-End Labeled 40-Nucleotide DNA Oligonucleotide. A 40-nucleotide (nt) DNA oligomer having the sequence 5′-CCC TCC CGA CTG CCT ATG ATG TTT ATC CTT TGG ATG GTC G-3′ was prepared on a Biosearch Model 8700 DNA synthesizer using phosphoramidite chemistry.[47] Also prepared was the complementary 40-nt sequence. 5′-End labeling of the DNA sequence shown above was carried out using 10 μg of the DNA in 50 μL of 50 mM Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 100 μM spermidine, 2.5 μM [γ-^{32}P] ATP (7000 Ci/mmol), and 500 units of T4 polynucleotide kinase (Bethesda Research Laboratories; 1 unit is defined as the amount of enzyme that will incorporate 1 nmol of labeled ATP into acid-precipitable material in 30 min). The reaction mixture was incubated at 37 °C for 1 h and then treated with 2.5 volumes of cold ethanol to effect precipitation of the DNA. The DNA was maintained at −80 °C for 12 h, recovered by centrifugation, and purified by gel electrophoresis on a 20% denaturing polyacrylamide gel.[32b] The recovered, 5′-^{32}P labeled DNA was used directly as a single-stranded substrate or else converted to the respective DNA duplex by annealing to its complementary 40-nt DNA oligomer (via heating in the presence of the complementary strand and 100 mM NaCl at 80 °C for 3 min, followed by slow cooling to 25 °C).

Cleavage of ^{32}P-End Labeled DNA by BLM Analogs. Reaction mixtures contained 2.5 pmol (~50 000 cpm) of 5′-^{32}P- or 3′-^{32}P-end labeled DNA and 200 μM sonicated calf thymus DNA in 10 μL of 10–20 mM Na cacodylate, pH 5.8 or 7.4. The reactions were initiated by the addition of the appropriate concentration of BLM and either Fe(III) + H_2O_2 or Fe(II) + dithiothreitol (DTT). The reaction mixtures were maintained at 25 °C for 1 h, quenched by the addition of 5 μL of loading buffer (10 M urea, 1.5 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), and applied to 20% polyacrylamide gels containing 8 M urea. Electrophoresis was carried out in 100 mM Tris-borate buffer, pH 8.4, containing 100 mM EDTA at 25 mA for 4 h. Autoradiography (Kodak XAR-2 film) was carried out at −80 °C for 24–48 h.

Preparation of a 3′-^{32}P-End Labeled 140-Nucleotide DNA Restriction Fragment. Plasmid pBR322 (12.5 μg) was digested simultaneously with 100 units each of *Hind* III and *Nci* I (Bethesda Research Laboratories; 1 unit is defined as that amount required to digest 1 μg of the substrate DNA in 1 h under the reaction conditions) in 50 μL of 0.5 M Tris-HCl, pH 8.0, containing 0.3 M NaCl, 0.1 M $MgCl_2$ and 1.0 mM dithiothreitol at 37 °C for 1 h. To this solution was added 125 μCi of [α-^{32}P]dATP and 100 units of AMV reverse transcriptase (Bethesda Research Laboratories; 1 unit incorporates 1 nmol of deoxynucleotide into acid-precipitable material in 10 min at 37 °C). The reaction mixture was incubated at 37 °C for 2 h. The 3′-^{32}P end-labeled 140-nt DNA fragment was purified on a nondenaturing 8% polyacrylamide gel and isolated by a crush and soak technique.

Relaxation of Supercoiled DNA by Bleomycin Analogs. Reactions were carried out in 16 μL (total volume) of 5 mM Na cacodylate buffer, pH 5.8, containing 200 ng of supercoiled pBR322 plasmid DNA and the appropriate concentration of Fe(III)·BLM analog and H_2O_2. Reaction mixtures were incubated at 25 °C for 1 h and then treated with 10 μL of loading buffer (40 mM Tris-OAc buffer, pH 7.8, containing 5 mM EDTA, 40% glycerol, 0.4% sodium dodecyl sulfate and 0.3% bromophenol blue) and applied to a 1.2% agarose gel containing 1 μg/mL of ethidium bromide. Horizontal gel electrophoresis was carried out in 40 mM Tris-OAc, pH 7.8, containing 5 mM EDTA at 130 V for 3–4 h (UV visualization).

Conclusions

Analogs of deglyco BLM, each of which lack one of the thiazole rings present in the parent molecule, were found to form Fe complexes that activated oxygen as well as BLM and deglyco BLM. Although both BLM monothiazole analogs effected the oxygenation and oxidation of low molecular weight substrates as well as BLM in what are believed to be bimolecular processes, neither could mediate sequence selective cleavage of DNA. We conclude that the bithiazole moiety is not required for Fe binding or oxygen activation, but that both rings must be present to mediate the sequence selective DNA cleavage characteristic of BLM group antibiotics.

Acknowledgment. We thank Dr. M. Kinter, University of Virginia, Mr. J. Blank, Pennsylvania State University, and Mrs. J. Iida, Shimadzu Corporation, for the high resolution mass spectra, and Dr. J. Shabanowitz, University of Virginia, for the LSIMS data. This work was supported by PHS Research Grants CA27603, CA38544, and CA53913 awarded by the National Cancer Institute.

(46) Carpino, L. A. *Acc. Chem. Res.* **1973**, *6*, 191.
(47) (a) Matteucci, M. D.; Caruthers, M. H. *J. Am. Chem. Soc.* **1981**, *103*, 3185. (b) McBride, L. J.; Caruthers, M. H. *Tetrahedron Lett.* **1983**, *24*, 245.

Correlating Backbone Amide and Side Chain Resonances in Larger Proteins by Multiple Relayed Triple Resonance NMR

Stephan Grzesiek† and Ad Bax*

Contribution from the Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892.
Received March 23, 1992

Abstract: A new three-dimensional triple resonance NMR experiment is described that correlates the amide ^{1}H and ^{15}N resonances of one residue simultaneously with both the $^{13}C_\alpha$ and $^{13}C_\beta$ resonances of its preceding residue. Sensitivity of the new experiment is comparable with that of the HN(CO)CA experiment (Bax, A.; Ikura, M. *J. Biomol. NMR* **1991**, *1*, 99–105), but the additional correlation to the C_β resonance of the preceding residue provides invaluable assignment information, previously inaccessible. The technique is demonstrated for interferon-γ, a homodimeric protein of 31.4 kDa, enriched uniformly with ^{13}C and ^{15}N.

The most difficult part in using the recently developed 3D triple resonance NMR for obtaining complete backbone assignments in larger proteins[1] involves the degeneracy in the H_α–C_α region of the ^{1}H–^{13}C shift correlation spectrum. Particularly for α-helical proteins with a narrow dispersion of H_α chemical shifts, this type of degeneracy frequently can make it difficult to establish in an unambiguous manner which backbone amide is correlated with which side chain. Even for the relatively well resolved spectrum of calmodulin, 40 such cases were reported.[1] Here we report a new 3D triple resonance technique that provides connectivity

† On leave from F. Hoffmann La Roche, Ltd., Basel, Switzerland.

(1) Ikura, M.; Kay, L. E.; Bax, A. *Biochemistry* **1990**, *29*, 4659–4667.

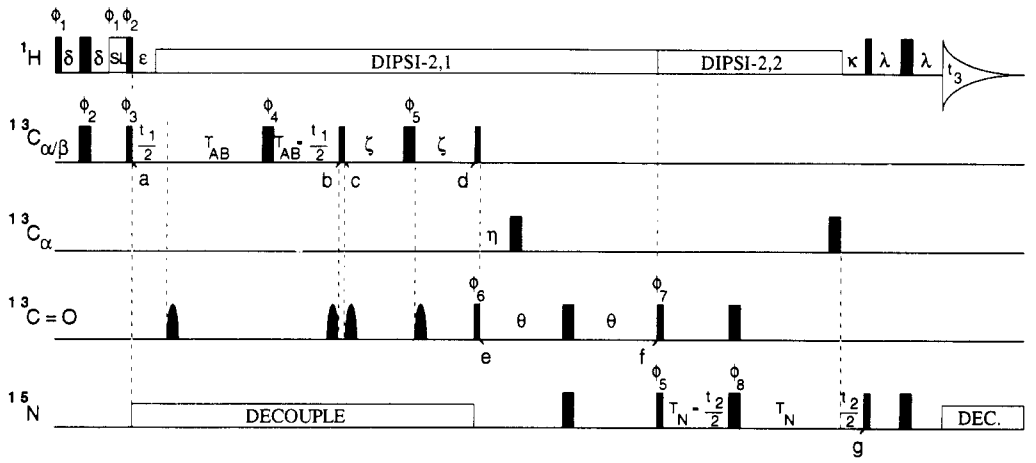

Figure 1. Pulse scheme of the CBCA(CO)NH experiment. Narrow pulses correspond to a 90° flip angle and wider pulses to 180°. Pulses for which no phase is indicated are applied along the x axis. The ^1H carrier is placed at 4.75 ppm until the 90_{ϕ_7} CO pulse is applied. After this time the ^1H carrier is switched to 8.1 ppm. The $C_{\alpha/\beta}$ carrier is positioned at 46 ppm and the power of the RF pulses is adjusted to yield zero excitation in the CO region (11.4-kHz RF for the 180° and 5.1-kHz RF for the 90° pulses at 151-MHz ^{13}C frequency). C_α pulses are applied at 56 ppm, using an RF field of 10.5 kHz. Rounded carbonyl pulses have a 180° flip angle and have the shape of the center lobe of a sin x/x function and a duration of 202 μs. The second and third shaped CO pulses serve to compensate the $C_{\alpha/\beta}$ phase errors caused by Bloch–Siegert effects[10] related to the first and fourth shaped pulse (see text). The rectangular CO pulses are applied using an RF field strength of 4.7 kHz, yielding minimal excitation of the C_α resonances. All ^{15}N pulses are applied with the carrier at 117 ppm, using a 6-kHz RF field. ^{15}N decoupling was accomplished using low power (1.5-kHz RF) WALTZ decoupling. ^1H decoupling during the magnetization relay is accomplished with a DIPSI-2 scheme,[18] using a 5.5-kHz RF field. Delay durations are $\delta = 1.5$ ms, $\epsilon = 2.3$ ms, $T_{AB} = 3.3$ ms, $\zeta = 3.7$ ms, $\eta = 4.5$ ms, $\theta = 11.4$ ms, $T_N = 11.1$ ms, $\kappa = 5.4$ ms, $\lambda = 2.25$ ms. The H_2O resonance was suppressed with the 1.8-ms purge pulse, SL. Phase cycling was as follows: $\phi_1 = y$; $\phi_2 = x,-x$; $\phi_3 = x$; $\phi_4 = 8(x),8(y),8(-x),8(-y)$; $\phi_5 = 4(x),4(-x)$; $\phi_6 = 2(x),2(-x)$; $\phi_7 = 51°$ (Bloch–Siegert phase error compensation); $\phi_8 = 8(x),8(-x)$; Rec. = $x,2(-x),x,-x,2(x),2(-x),2(x),-x,x,2(-x),x$. Quadrature in F_1 and F_2 is obtained by altering ϕ_3 and ϕ_5 in the usual manner.[2]

information between the amide ^1H and ^{15}N of one residue and both the $^{13}C_\alpha$ and $^{13}C_\beta$ resonances of its preceding residue. Consequently, this experiment removes the need for recording a HN(CO)CA type correlation[2] and in addition provides the crucial J connectivity between backbone and side chain resonances.

Experimental Section

A form of interferon-γ from which the last 10 residues are missing (IFN-γΔ10) shows the highest biological activity.[3] IFN-γΔ10, enriched uniformly (>98%) with ^{15}N and ^{13}C, was obtained from *E. coli*. X-ray crystallographic work has shown that the protein is highly α-helical and exists as a highly intertwined dimer.[4] The total molecular weight of the (unlabeled) dimer is 31 410. Details regarding the expression and purification of the protein are presented elsewhere.[3,5] NMR experiments were carried out on a sample containing 11 mg of IFN-γ in 0.45 mL of 95% H_2O/5% D_2O, pH 6.2.

Experiments were carried out at 600-MHz ^1H frequency on a Bruker AMX-600 spectrometer, equipped with an external 150 W class A/B poweramplifier for ^{13}C and operating with software version 911101. The 3D CBCA(CO)NH spectrum was obtained from a $52^*(t_1) \times 32^*(t_2) \times 512^*(t_3)$ data set, with acquisition times of 6.16 ms (t_1), 20.0 ms (t_2), and 55.3 ms (t_3). To implement the pulse program on our AMX spectrometer, t_1 increments with $t_1/2 < \epsilon$ (Figure 1) were executed with a different section of the total pulse program compared to values of $t_1/2 > \epsilon$. The total measuring time was 2.5 days. Mirror image linear prediction[6] in both the t_1 and t_2 domain was used, and data were zero filled to yield a 256 × 128 × 1024 matrix for the absorptive part of the final 3D spectrum.

Results and Discussion

The pulse scheme used in the present work is sketched in Figure 1. A detailed description of an analogous experiment will be described elsewhere,[7] and here we will outline only the general mechanism of the CBCA(CO)NH experiment. The scheme starts with an INEPT transfer to enhance the polarization of ^{13}C. Between time points *a* and *b* the aliphatic magnetization is subjected to a constant-time evolution period[7-9] of duration $1/(4J_{CC})$ ($2T_{AB} \sim 7$ ms). Scalar coupling to the carbonyl carbon is removed by the (shaped) 180° pulse applied $t_1/2$ after time *a*. Note that during the application of this ^{13}CO pulse the $^{13}C_\alpha$ changes its resonance frequency due to the Bloch–Siegert effect.[10] The "phase error" resulting from this Bloch–Siegert effect, which is subsequently inverted by the $180°_{\phi_4}$ $C_{\alpha/\beta}$ pulse, can be easily eliminated by applying the same shaped ^{13}CO pulse at the end of the constant-time evolution period, immediately prior to time *b*. This ensures a pure cosine modulation in the t_1 dimension and thus facilitates phasing in the F_1 dimension of the final spectrum. Similarly, the Bloch–Siegert phase error caused by the fourth shaped ^{13}CO pulse is compensated for by the third pulse.

At time *b*, the 90° $C_{\alpha/\beta}$ pulse transfers magnetization from C_β to C_α. A fraction ($\cos(2\pi J_{C_\alpha C_\beta} T_{AB})$) of the magnetization that started at C_α remains on C_α after this 90° pulse (time *c*). At the end of the subsequent period of total duration 2ζ, between time points *d* and *e*, C_α magnetization is transferred to the adjacent carbonyl nucleus, using the relatively large $^1J_{C_\alpha CO}$ coupling. Carbonyl carbon magnetization refocuses with respect to its attached $^{13}C_\alpha$ spin during the period 2η and dephases with respect to its adjacent ^{15}N spin during the time 2θ. At time *f* this antiphase ^{13}CO magnetization is transferred to its directly bonded ^{15}N nucleus. During the second constant-time evolution period, of total duration $2T_N$ (22 ms), antiphase ^{15}N magnetization rephases with respect to its coupled ^{13}CO nucleus and dephases with respect to its directly attached ^1H spin during the final fraction κ, when ^1H decoupling is switched off. At time *g*, this ^{15}N magnetization (antiphase with respect to H_N) is transferred to its directly attached ^1H by means of a reverse INEPT transfer, prior to detection during the time t_3. For proteins in the 20–30-kDa range, all dephasing and rephasing intervals needed for the transfer of magnetization in the CBCA(CO)NH experiment are relatively short compared to the pertinent transverse relaxation times T_2. Therefore, the efficiency of the pulse scheme is quite high, despite the relatively

(2) Ikura, M.; Bax, A. *J. Biomol. NMR* **1991**, *1*, 99–104.
(3) Döbeli, H.; Gentz, R.; Jucker, W.; Garotta, G.; Hartmann, D. W.; Hochuli, E. *J. Biotechnol.* **1988**, *7*, 199–216.
(4) Ealick, S. E.; Cook, W. J.; Vijay-Kumar, S.; Carson, M.; Nagabhushan, T. L.; Trotta, P. P.; Bugg, C. E. *Science* **1991**, *252*, 698–702.
(5) Grzesiek, S.; Döbeli, H.; Gentz, R.; Garotta, G.; Labhardt, A. M.; Bax, A. *Biochemistry*, submitted for publication.
(6) Zhu, G.; Bax, A. *J. Magn. Reson.* **1990**, *90*, 405–410.
(7) Grzesiek, S.; Bax, A. *J. Magn. Reson.*, submitted for publication.
(8) Bax, A.; Freeman, R. *J. Magn. Reson.* **1981**, *44*, 542–561.
(9) Palmer, A. G., III; Fairbrother, W. J.; Cavanagh, J.; Wright, P. E.; Rance, M. *J. Biomol. NMR* **1992**, *2*, 103–108.
(10) Freeman, R. *A Handbook of Nuclear Magnetic Reson[illegible]e*; Wiley: New York, 1988; pp 14–16.

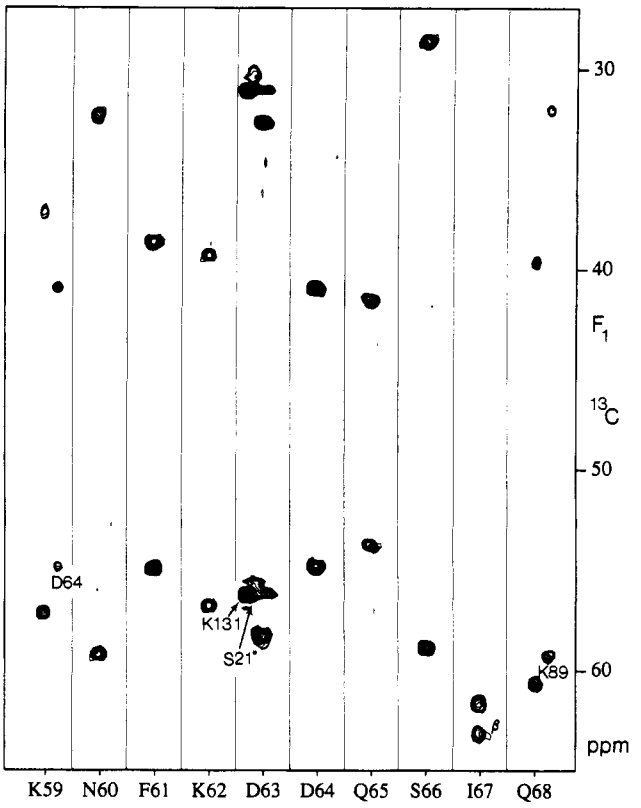

Figure 2. F_1 (C_α, C_β) strips for residues K59–Q68, taken from the 600-MHz 3D CBCA(CO)NH spectrum of interferon-γ at the $^1H/^{15}N$ (F_3/F_2) frequencies of their amides. In the leftmost strip, the amide of K59 correlates with C_α and C_β of F58. Also visible in this strip is the correlation for the amide of D64 (which has a ^1H–^{15}N correlation very close to that of K59) to the C_α and C_β of D63. These latter correlations are observed with much higher intensity in the sixth strip.

large number of RF pulses required for the magnetization transfer.

Figure 2 demonstrates the application of the CBCA(CO)NH method to the protein interferon-γ, a homodimer of 31.4 kDa. Experiments were recorded at 27 °C, and at this temperature the ^1H and ^{13}C line widths for residues with little internal mobility are ~40–50 Hz. Several of the recently published 3D experiments did not yield satisfactory results[5] because of these large line widths and due to the severe overlap in the H_α–C_α region of the ^1H–^{13}C shift correlation spectrum (supplementary material). Figure 2 shows F_1 strips from the 3D spectrum, taken at the amide $^1H/^{15}N$ frequencies of ten consecutive residues, located in the hydrophobic core of the protein. These results clearly illustrate the power and effectiveness of the new experiment. For each amide, both the C_α and C_β chemical shifts of the preceding amino acid are identified. Note that during the initial sequential assignment stage the C_β chemical shift is particularly useful for determining the type of amino acid, greatly facilitating the task of making sequence specific assignments.

The present experiment provides the first example of utilizing the relatively large and uniform one-bond J couplings for multiple relayed J connectivity between backbone amide and side chain resonances in proteins. For small proteins, similar kinds of connectivity can be obtained using multiple ^1H–^1H relay[11] or HOHAHA/TOCSY experiments.[12,13] In larger proteins these J_{HH} based experiments are usually unsuccessful, particularly if J_{HNH_α} is small. In favorable cases connectivity between the amide $^1H/^{15}N$ and the side chain H_β can be obtained via the $^3J_{NH_\beta}$ or $^3J_{COH_\beta}$ coupling,[14-16] but in practice these experiments fail for proteins the size of interferon-γ or if these couplings are small.

The CBCA(CO)NH experiment provides a very robust and efficient method for obtaining amide to side chain J connectivity. The scheme can be readily modified to yield connectivity to the H_α and H_β protons by introducing an evolution period prior to the first INEPT transfer and by replacing the first constant-time evolution period by a fixed interval (i.e., by keeping $t_1 = 0$ in the scheme of Figure 1). Another potentially useful modification shifts the second constant-time evolution period from ^{15}N to ^{13}CO. An experiment analogous to the CBCA(CO)NH technique described here, which attempts to correlate the C_α and C_β resonances directly with the intraresidue amide ^1H and ^{15}N resonances via J_{NC_α}, was unsuccessful for IFN-γ, due to the large C_α line width relative to the J_{NC_α} coupling. However, for smaller proteins this latter experiment[7] provides a powerful complement to the CBCA(CO)NH experiment.

In principle, the amide $^1H/^{15}N$ could also be correlated simultaneously with both H_β and C_β resonances by transforming the dephasing interval 2δ in Figure 1 into a constant-time evolution period and recording the spectrum in a 4D manner. The pulse scheme could be executed even in a 5D or 6D manner by replacing the fixed rephasing/dephasing delays 2ζ and 2θ by "constant-time" evolution periods. However, for practical reasons such increases in dimensionality are clearly undesirable.[17]

Acknowledgment. We thank Drs. R. Gentz, H. Döbeli, W. Vetter, W. Meister, M. Zulauf, and W. Klaus (Hoffman-LaRoche, Basel) for providing us with purified $^{13}C/^{15}N$ enriched interferon-γ and its characterization by biophysical techniques. S.G. acknowledges funding by the ROCHE Research Foundation and thanks Dr. A. M. Labhardt for enthusiastic support. This work was supported by the Intramural AIDS Targeted Anti-Viral Program of the Office of the Director of the National Institutes of Health.

Supplementary Material Available: One figure displaying the H_α–C_α region of the ^1H–^{13}C shift correlation spectrum of interferon-γ (1 page). Ordering information is given on any current masthead page.

(11) Eich, G.; Bodenhausen, G.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 3731–3732.
(12) Braunschweiler, R. R.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521–528.
(13) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355–360.
(14) Chary, K. V. R.; Otting, G.; Wüthrich, K. *J. Magn. Reson.* **1991**, *93*, 218–224.
(15) Archer, S. J.; Ikura, M.; Torchia, D. A.; Bax, A. *J. Magn. Reson.* **1991**, *95*, 636–641.
(16) Grzesiek, S.; Ikura, M.; Clore, G. M.; Gronenborn, A. M.; Bax, A. *J. Magn. Reson.* **1992**, *96*, 215–221.
(17) Bax, A.; Grzesiek, S. *Acc. Chem. Res.*, submitted for publication.
(18) Shaka, A. J.; Lee, C. J.; Pines, A. *J. Magn. Reson.* **1988**, *77*, 274–293.