Kyogoku. 1985. Phosphorus-31 nuclear magnetic resonance of highly oriented DNA fibers. Static geometry of DNA double helices. *Biochemistry*. 24:887–895.
26. Fujiwara, T., and H. Shindo. 1985. Phosphorus-31 nuclear magnetic resonance of highly oriented DNA fibers. Molecular motions in hydrated DNA. *Biochemistry*. 24:896–902.
27. Kearns, D. 1984. NMR Studies of conformational states and dynamics of DNA. *CRC Crit. Rev. Biochem.* 15:237–290.
28. Opella, S., W. Wise, and J. DiVerdi. 1981. Deoxyribonucleic acid dynamics from phosphorus-31 nuclear magnetic resonance. *Biochemistry*. 20:284–290.
29. Clore, G., and M. Gronenborn. 1984. Internal mobility in a double-stranded B DNA hexamer and undecamer. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 172:219–225.
30. Feigon, J., W. Denny, W. Leupin, and D. Kearns. 1983. Proton nuclear magnetic resonance investigation of the conformation and dynamics in the synthetic deoxyribonucleic acid decamers d(ATATCGATAT) and d(ATATGCATAT). *Biochemistry*. 22:5930–5942.
31. Ephrussi, A., G. Church, S. Tonegawa, and W. Gilbert. 1985. B lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. *Science (Wash. DC)*. 227:134–140.

PROTEIN-PROTEIN INTERACTIONS IN DNA RECOGNITION

^{1}H-Nuclear Magnetic Resonance Studies of λ cI Repressors Genetically Altered by Site-Directed Mutagenesis

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Phage λ repressor contains two domains separable by proteolysis (1). The isolated NH_2-terminal domain recognizes operator DNA and provides a tractable model system for biophysical and genetic studies of macromolecular recognition (2–5). The active species in operator recognition is a dimer. We describe here one- and two-dimensional ^{2}H-NMR studies of the dimer interface in wild-type and genetically altered repressors.

The structure of the NH_2-terminal domain, a fragment of 92 residues, has been determined by x-ray crystallography (6). As shown in Fig. 1 *A*, the domain consists of five α helices. The first four form a globular region, which

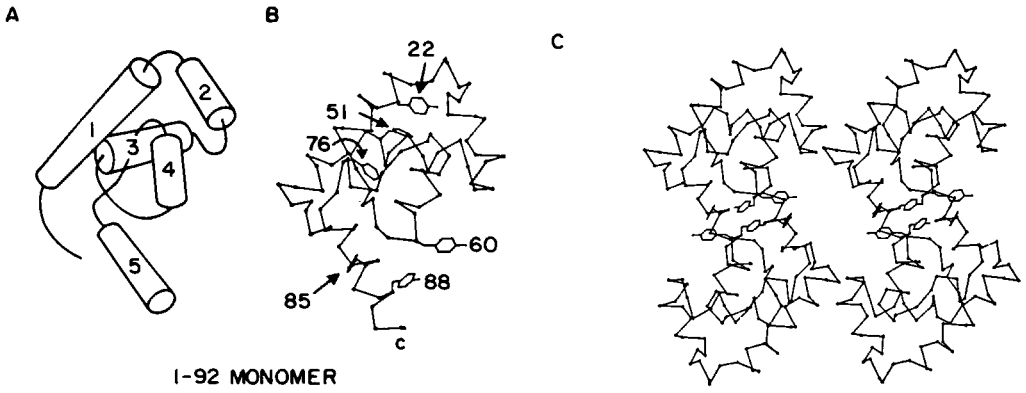

FIGURE 1 (*A*) Model of the 1–92 domain of phage λ repressor. The α helices are shown as cylinders. The first four helices form a globular region, and the fifth extends outward. (*B*) α-carbon representation showing the aromatic side chains. Tyr22 and Phe51, and Phe76 are buried in the hydrophobic interior and provide sensitive markers for changes in tertiary structure. Tyr60, Tyr85, and Tyr88 are on the surface of the monomer structure. Their environments are altered in the dimer. (*C*) Stereo pair, showing the helix 5–helix 5 interaction. This helix is amphipathic, and the interaction is primarily hydrophobic. In the crystallographic dimer the two DNA-binding surfaces are appropriately oriented to contact successive major grooves of B-DNA. The structures were calculated from the crystal coordinates of Pabo and Lewis (6).

contains the DNA-binding surface, and the fifth extends outward. In the crystallographic dimer, helix 5 packs against the symmetry-related helix 5 from the other subunit, as shown in Fig. 1 *C*.

In the absence of DNA, the NH_2-terminal fragment forms a dimer only at high protein concentration ($K_d \sim 10^{-4}$ M) (unpublished results). Because operator binding and dimerization are coupled equilibria, dimerization contributes to apparent operator affinity (3, 4). Conversely, mutations that stabilize the dimer should increase operator affinity. Because the strongest dimer contact would be covalent, Pabo and Suchanek developed modeling methods to determine possible sites for intermolecular disulfide bonds.[1] There are no cysteines in the wild-type NH_2-terminal domain (7), but these may be introduced by site-directed mutagenesis. Two types of disulfide bonds may be envisaged. A "correct" disulfide would yield a covalent dimer whose structure would be similar to the wild-type, so that two DNA-binding surfaces would remain appropriately oriented to interact with operator DNA. Alternatively, one can imagine an "incorrect" disulfide that would cross-link the dimer in a non-native configuration, reducing operator affinity.

Fig. 2 shows the native NH_2-terminal dimer and the position of a correct disulfide suggested by Pabo and Suchanek.[1] If the native conformation is retained in the mutant (8), the symmetry-related cysteines would be properly positioned to form an intermolcular disulfide. Because Tyr85 and Tyr85′ are distant in the native dimer structure deduced from the crystallographic results, the analogous Cys85 mutant would not be expected readily to form a covalent dimer. Moreover, formation of the "incorrect" Cys85 disulfide could distort the native quaternary structure. These design features are represented schematically in Fig. 3.

The Cys85 and Cys88 mutations were introduced into the NH_2-terminal domain (residues 1–102) by directed mutagenesis, purified, and characterized.[2] It was observed that the Cys88 domain spontaneously forms a covalent dimer, as suggested by Pabo and Suchanek. As expected for a correct disulfide-bonded dimer, the covalent dimer is at least 10-fold more active in operator DNA binding than the wild-type domain. The Cys85 domain does not readily form a covalent dimer; its reduced form has wild-type activity. A Cys85 disulfide can be formed, following reaction with 5,5′-dithio-bis-1-nitrobenzoic acid (DTNB) (9), and this species binds operator DNA at least 10-fold less well than wild-type.

[1]Pabo, C. O., and E. Suchanek, Computer-aided model building strategies for protein design. *Proc. Natl. Acad. Sci. USA.* Manuscript submitted for publication.

[2]Sauer, R. T., R. Stearman, K. Hehir, E. Suchanek, and C. O. Pabo. Manuscript in preparation.

RESULTS

The aromatic region of the ^{1}H-NMR spectrum of the wild-type fragment is shown in Fig. 4 *A*. The large dispersion in chemical shifts is the result of the native structure of the NH_2-terminal domain. As shown in Fig. 1 *B*, Tyr22, Phe51, and Phe76 are buried in the hydrophobic interior of the NH_2-terminal domain. Because of their unusual local magnetic environments, these internal aromatic resonances provide sensitive markers for tertiary structure (5). Quaternary interactions influence the environments of Tyr60, Tyr85, and Tyr88, as illustrated in Fig. 1 *C*. Their resonances provide sensitive markers for the structure and dynamics of the dimer interface. Tertiary and quaternary nuclear Overhauser effects involving these aromatic resonances are shown in Fig. 5.

The aromatic spectrum of the reduced form of Tyr85 → Cys mutant domain is shown in Fig. 4 *B*. The native structure of the reduced Cys85 domain, as inferred from its ^{1}H-NMR spectrum, is in accord with its wild-type level of activity.[2] The aromatic spectrum of the Cys85 disulfide-bonded dimer is shown in Fig. 4 *C*. As discussed in the legend, this spectrum suggests a major change in the dimer contact, with preservation of tertiary structure. Because the individual DNA-binding surfaces are intact, the decreased operator affinity exhibited by this species may be attributed to inappropriate subunit orientation. The aromatic spectrum of the Cys88 disulfide-bonded dimer is shown in Fig. 4 *D*. Its tertiary structure is also not significantly perturbed. In monitoring the dimerization of the wild-type and Cys85 domains, Tyr88 provided the major structural marker. As shown in Fig. 1 *C*, Tyr88 is stacked against Tyr88′ in the dimer interface; its unusual local magnetic environment in the dimer is sensitive to perturbations in quaternary structure. In its absence, the nature of the dimer contact is difficult to evaluate. In addition, shifts observed in the resonances of Tyr60 and Tyr85 are difficult to interpret, because removal of Tyr88 can create ring-current as well as structural perturbations. Nevertheless, the increased operator affinity exhibited by the Cys88 disulfide-bonded dimer suggests that a native-like quaternary structure is retained.[2]

The structural effects of the Cys85 and Cys88 disulfide bonds may be indirectly probed through measurements of thermal stability. The wild-type NH_2-terminal domain denatures near 50°C (1). This transition is irreversible at high concentrations, and the unfolded state is an aggregate that is poorly characterized (10). Thermal denaturation may be monitored by ^{1}H-NMR (data not shown). Between 46° and 50° all the aromatic resonances of the wild-type domain (except those near the random-coil frequencies of Tyr101) are observed to broaden and disappear. This NMR transition is consistent with measurements by differential scanning calorimetry, circular dichroism and protease sensitivity (1, 10), and reflects the aggregation-coupled denaturation of the domain.

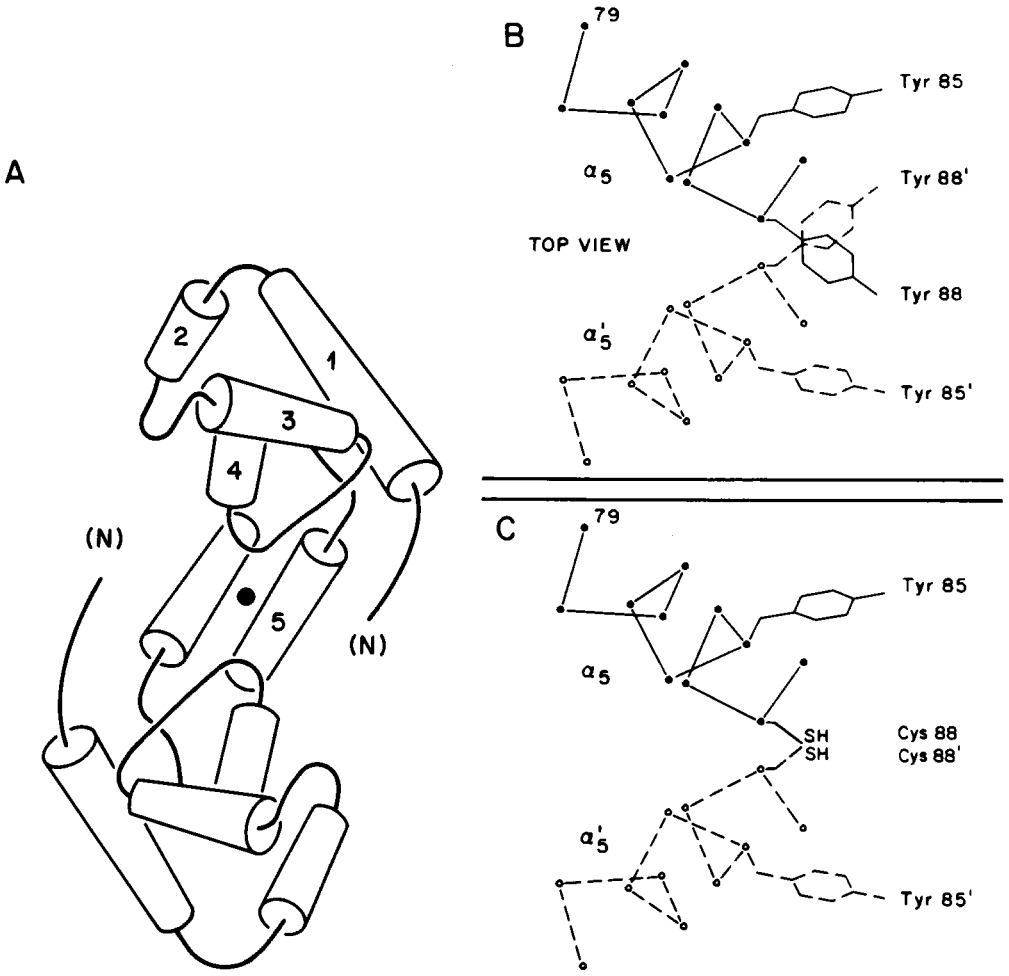

FIGURE 2 (*A*) Cylinder model of the crystallographic dimer (front view). The symmetry axis is orthogonal to the plane of the figure. (*B*) Top view of the helix 5–helix 5 interaction. The upper helix (solid line) is projecting into the plane of the figure; the lower helix (dashed line) is projecting out of the plane of the figure. The symmetry axis is horizontal. Tyr88 is stacked against Tyr88′ and also interacts with Tyr85′. Because Tyr88 and Tyr88′ are symmetry-related, their ^{1}H-NMR resonances cannot be distinguished. A nuclear Overhauser effect is observed between Tyr88 and Tyr85′ (equivalently, between Tyr88′ and Tyr85), as shown in Fig. 5. (*C*) The amino-acid substitution Tyr88 → Cys was predicted by Pabo and Suchanek to allow formation of an intermolecular disulfide bond without inducing nonlocal structural perturbations.[1] The analogous Cys85 mutant would not be expected readily to form a covalent dimer.

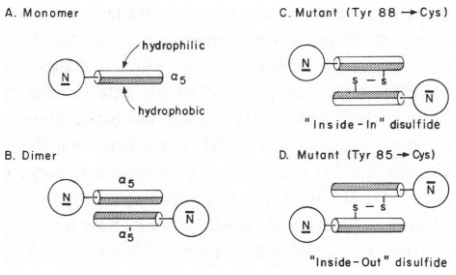

FIGURE 3 Schematic representation of the dimer contact. (*A*) The sphere (*N*) represents the globular portion of the NH_2-terminal domain, containing the first four helices. The cylinder represents helix 5, which is amphipathic. (*B*) The native dimer contact may be viewed as a hydrophobic interaction between amphipathic helices. The Cys88 intermolecular disulfide bond (*C*) was designed by Pabo and Suchanek to preserve the native dimer structure ("inside-in"). The Cys85 disulfide-bonded dimer (*D*) was predicted to distort it.[1] In the native structure Tyr85 and Tyr85′ are nearly on opposite surfaces, and shown in Fig. 1 *C*. The Cys85 covalent dimer ("inside-out") was found to be at least 10-fold less active in operator-binding than either wild-type or the reduced form.[2] These models assume that the dimer contact (cylinder) and globular region (sphere) are structurally distinct and may be independently manipulated. Although our ^{1}H-NMR results support this assumption, these models are schematic only. The actual structure of the Cys-85–Cys-85 dimer is not known. Indeed, it is not known where helix 5 remains as an α-helix in the incorrectly disulfide-bonded species.

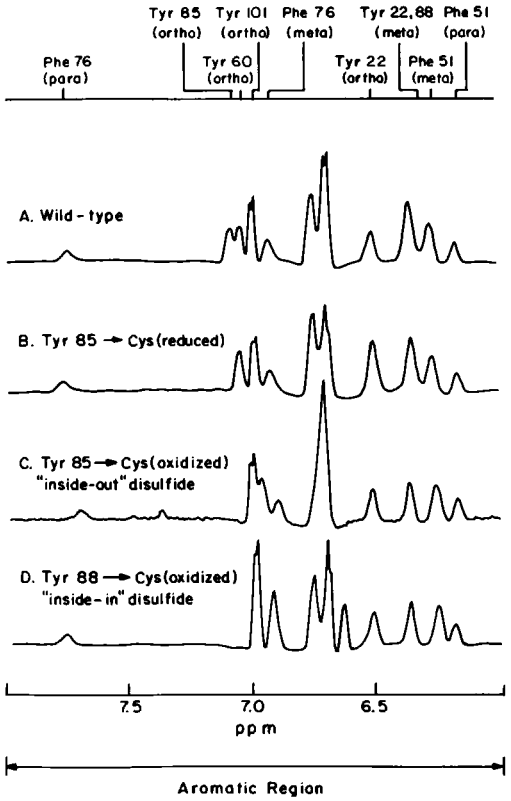

FIGURE 4 (*A*) Aromatic spectrum of the wild-type 1–102 domain at 30°C and 500 MHz. The fragment is dimeric at the concentration and conditions used. The large dispersion in chemical shifts is the result of the native structure of the NH_2-terminal domain. In the primary sequence there are seven aromatic groups: Tyr22, Phe51, Tyr60, Phe76, Tyr85, Tyr88, and Tyr101 (7). Their resonances provide sensitive markers for changes in tertiary and quaternary structure. Ring-current shift calculations suggest that their secondary shifts should be sensitive to displacements as small as 0.2 Å (5, 11, 12). Tyr88 is partially buried in the dimer interface, where it stacks against Tyr88′ from the other subunit. Its resonances are shifted upfield by this interaction. Although its meta protons are equivalent, its ortho resonances are in intermediate exchange, indicating that there are significant barriers to ring rotation in this environment. Tyr85 is on the surface of the dimer, but is near Tyr-88′. An intermolecular nuclear Overhauser effect is observed between the Tyr85 and Tyr88′ rings (equivalently, between Tyr85′ and Tyr88), as shown in Fig. 5. Tyr60 is also on the surface of the dimer, and may be near COOH-terminal residues from the other subunit. The protein concentration was 5 mM, assuming that a 1 mg/ml solution has an absorbance of 0.61/cm at 280 nm. The spectrum is the sum of 200 transients with recycle delay of 4 s. A convolution difference with parameters GM4, EM20, and subtraction factor 1.0 was applied before Fourier transformation. (*B*) Aromatic spectrum of the reduced form of the Cys85 mutant domain. The Tyr85 spin system is absent, consistent with the amino-acid substitution. The resonances of Tyr22, Phe51, and Phe76 are essentially identical to those of wild-type (relative shifts <0.03 ppm). This indicates that the tertiary structure of the domain is not affected by the mutation. The ortho resonance of Tyr88, however, is now in fast (rather than intermediate) exchange. This resonance, which overlaps the ortho resonance of Tyr22, is indicated by an asterisk. The change in exchange character implies either an increase in the rate of ring rotation or a decrease in the local magnetic inequivalence. The Tyr88 meta resonance is not perturbed. The aromatic spectrum is not altered by the addition of 15 mM β-mercaptoethanol or dithiothreitol as a reducing agent. The protein concentration was 5 mM. The spectrum is the sum of 216 transients with recycle delay of 4 s. A convolution difference with parameters GM4, EM20, 1.0 was applied. (*C*) Aromatic spectrum of the Cys85 covalent dimer. The protein concentration was 2 mM. The resonance of Tyr 22 and Phe51 are virtually unperturbed (relative shifts <0.02 ppm), indicating that the individual DNA-binding surfaces are intact. Dramatic changes are observed elsewhere in the spectrum. The Tyr88 resonances are absent in the upfield region, suggesting that its stacked geometry has been disrupted. Although the resonances of Tyr88, Tyr85, and Tyr60 have not been assigned in the mutant spectrum, they have clearly been perturbed by the formation of the disulfide bond. Phe76 exhibits small shifts (0.03 ppm). These may reflect a change in the configuration of the loop between helix 4 and helix 5. The spectrum is the sum of 1,000 transients with recycle delay of 2 s. A convolution difference with parameters GM5, EM30, 1.0 was applied. (*D*) Aromatic spectrum of the Cys88 covalent dimer. The Tyr88 spin system is absent, consistent with the amino-acid substitution. The resonances of Tyr22, Phe51, and Phe76 are almost identical to those of wild-type (relative shifts <0.3 ppm), indicating as above a correspondence of tertiary structure. The protein concentration was 5 mM. The spectrum is the sum of 604 transients with recycle delay of 1.5 s. A convolution difference with parameters GM4, EM20, 1.0 was applied. In all acquisitions the residual HOD signal was presaturated for 1 s. The proteins were dissolved in a buffer containing 200 mM KCl, 50 mM potassium phosphate (pD 7. 4), 1 mM sodium azide, and 0.1 mM EDTA in 99.9% D_2O.

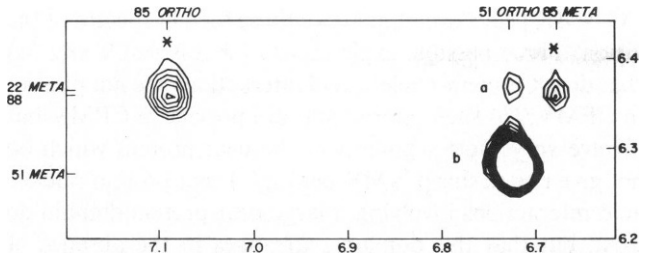

FIGURE 5 Portion of the aromatic region of the two-dimensional pure-phase nuclear Overhauser effect spectrum (13) of the wild-type 1–102 dimeric domain. Cross-peak *a* shows an effect between Tyr22 and Phe51, which are stacked in the interior of the domain. Asterisks indicate effects between Tyr85 and Tyr88. In the crystal structure, Tyr88 comes within 4 Å of Tyr85′. Cross-peak *b* is a very large Overhauser effect between the ortho and meta protons on the same ring, Phe51. 1,024 points were sampled over a sweep width of 5,000 Hz. 96 scans were acquired per t_1 value, and 220 T_1 values were obtained. The mixing time was 200 ms. The data matrix was zero-filled to 1,024 × 1,024. A convolution difference with parameters GM3, EM20, 1.0 was applied in both dimensions. The final data matrix was made symmetric by minimum value.

Under these conditions the Cys88 disulfide-bonded dimer denatures between 56° and 60°C (data not shown). The Cys85 disulfide-bonded dimer denatures between 36° and 40°C (data not shown). Thus, a correct disulfide appears to stabilize the protein, and an incorrect disulfide to destabilize it.

We thank C. Pabo, E. Suchanek, and K. Hehir for mutant plasmids and communication of unpublished results; C. Pabo and M. Lewis for crystal coordinates; and D. States, D. Ruben, and J. Hoch for NMR software.

This work was supported by grants from the National Institutes of Health (NIH) to M. Karplus (GM-30804) and R. T. Sauer (AI-15706). 500 MHz spectra were recorded at the High Field NMR Resource of the Francis Bitter National Magnet Laboratory (NIH, RR-00995).

Received for publication 8 May 1985 and in revised form 24 June 1985.

REFERENCES

1. Pabo, C. O., R. T. Sauer, J. M. Sturtevant, and M. Ptashne. 1979. The λ repressor contains two domains. *Proc. Natl. Acad. Sci. USA.* 76:1608–1612.
2. Sauer, R. T., C. O. Pabo, B. J. Meyer, M. Ptashne, and K. C. Backman. 1979. Regulatory functions of the λ repressor reside in the amino terminal domain. *Nature (Lond.).* 279:396–400.
3. Nelson, H. C. M., M. Hecht, and R. T. Sauer. 1983. Mutations defining the operator-binding sits of bacteriophage λ repressor. *Cold Spring Harbor Symp. Quant. Biol.* 47:441–449.
4. M. Hecht, H. C. M. Nelson, and R. T. Sauer. 1983. Mutations in λ repressor's amino-terminal domain: implications for protein stability and DNA binding. *Proc. Natl. Acad. Sci. USA.* 80:2676–2680.
5. Weiss, M. A., R. T. Sauer, D. J. Patel, and M. Karplus. 1984. Amino-terminal arm of the λ repressor: a ^{1}H-NMR study. *Biochemistry.* 23:5090–5095.
6. Pabo, C. O., and M. Lewis. 1982. The operator-binding domain of λ repressor: structure and DNA recognition. *Nature (Lond.).* 298:443–447.
7. Sauer, R. T., and R. Anderegg. 1978. Primary structure of the λ repressor. *Biochemistry.* 17:1092–1100.
8. Shih, H. H.-L., J. Brady, and M. Karplus. 1985. Structure of proteins with single-site mutations: a minimum perturbation approach. *Proc. Natl. Acad. Sci. USA.* 1697–1701.
9. Means, G. E., and R. E. Feeney. 1971. Chemical Modification of Proteins. Holder-Day, Inc., San Francisco.
10. Hecht, M. H., J. M. Sturtevant, and R. T. Sauer. 1984. Effect of single amino-acid replacements on the thermal stability of the NH_2-terminal domain of phage λ repressor. *Proc. Natl. Acad. Sci. USA.* 81:5685–5689.
11. Johnson, C. E., and F. A. Bovey. 1958. Calculation of nuclear magnetic resonance spectra of aromatic hydrocarbons. *J. Chem. Phys.* 29:1012–1014.
12. Hoch, J. C., C. M. Dobson, and M. Karplus. 1981. Fluctuations and averaging of proton chemical shifts in the bovine pancreatic trypsin inhibitor. *Biochemistry.* 21:1118–1125.
13. States, D. J., R. A. Haberkorn, and D. J. Ruben. 1982. A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. *J. Magn. Reson.* 48:286–292.

NUCLEAR MAGNETIC RESONANCE STUDIES OF SPHERICAL PLANT VIRUSES

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We have used nuclear magnetic resonance (NMR) spectroscopy to study protein-nucleic acid interactions and polyamine exchangeability in belladonna mottle virus (BDMV), turnip yellow mosaic virus (TYMV), and cowpea mosaic virus (CPMV). The protein shells of BDMV and TYMV each are composed of 180 copies of a 22,000 d protein; each capsid holds one single-stranded RNA molecule of ~2.0×10^6 d. CPMV, which has a divided genome, consists of two single-stranded RNA molecules packaged separately in identical capsids; the capsid is composed of 60 copies each of two different proteins (22,000 d and 42,000 d). Capsids (devoid of RNA) occur naturally for all the three viruses. The diameters of these virions and their capsids are each ~280 Å. Our results demonstrate that ^{1}H and ^{13}C NMR can be used to observe polyamines (such as spermidine) in a virus particle and to study their effect on the dynamics of capsid groups and overall virus stability. In BDMV and TYMV, a mobile peptide domain, whose NMR signal is easily resolved in spectra of the empty capsids, interacts with the RNA. The capsid of CPMV, by contrast, shows no evidence for such a highly mobile domain.