Dynamic filtering by two-dimensional $^1$H NMR with application to phage $\lambda$ repressor
(protein–DNA interactions/protein dynamics/segmental flexibility)

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ABSTRACT Flexible regions of proteins play an important role in catalysis, ligand binding, and macromolecular interactions. Because of its enhanced sensitivity to motional narrowing, two-dimensional coupling constant J-correlated $^1$H NMR may be used to observe these regions selectively. Dynamic filtering is an intrinsic feature of this experiment because cross-peak amplitude decays rapidly as linewidths approach the coupling constant. We demonstrate here the flexibility of the NH$_2$-terminal arm of phage $\lambda$ repressor, which is thought to wrap around the double helix in the repressor–operator complex. The assignment of arm resonances is made possible by the construction of mutant repressor genes containing successive NH$_2$-terminal deletions.

Phage $\lambda$ repressor regulates gene expression by binding to specific operator sequences in the phage genome (1). The determination of the crystal structure of its operator-binding domain has focused attention upon the molecular details of protein–DNA recognition (2–4). This domain, consisting of the first 92 residues, contains five $\alpha$ helices and an extended NH$_2$-terminal arm. The former fold into a globular domain whose second and third helices are thought to interact with the operator. Homologous helices are observed also in a number of other DNA-binding proteins and may provide a general scaffold for operator recognition (5–10). The NH$_2$-terminal arm (NH$_2$-Ser-Thr-Lys-Lys-Lys-Pro–) is thought to wrap around the double helix to contact the back of the operator site (11) as illustrated in Fig. 1. Flexibility of the arm is an essential feature of this model, since otherwise there would be large kinetic barriers to operator binding and release.

We report here a new application of two-dimensional NMR that directly demonstrates the flexibility of the NH$_2$-terminal arm of intact phage $\lambda$ repressor. The coupling constant J-correlated pulse sequence (12, 13, §) is shown to be a dynamic filter, enabling the selective observation of mobile regions of macromolecules. By coincidence of macromolecules, this filter attenuates signals from spins whose transverse relaxation is dominated by macromolecular rotation. Accordingly, the correlated spectrum of $\lambda$ repressor contains only a few spin systems. These must belong to residues that are reorienting more quickly than the rate of overall rotation. Most of these resonances are rigorously assigned to the NH$_2$-terminal arm through the study of mutant repressors containing successive NH$_2$-terminal deletions.

MATERIALS AND METHODS

Wild-type phage $\lambda$ repressor was purified as described (14, 15). Mutant repressor genes (cl) containing successive NH$_2$-terminal deletions were constructed and cloned as described in the legend to Fig. 2. In-frame deletions either were selected by immunity to bacteriophage $\lambda$ or were screened (in the case of 7–236) for the introduction of an Hpa I restriction site. Such clones were screened by NaDodSO$_4$/PAGE electrophoresis for overproduction of a 26-kDa protein. Candidates were then sequenced by the method of Maxam and Gilbert (19). Clones beginning with residues 2, 4, and 7 were identified. We will refer to these mutant repressors as 2–236, 4–236, and 7–236, respectively (wild-type repressor contains 236 residues). The mutant proteins were purified essentially as wild-type protein was. NH$_2$-terminal protein sequencing of 4–236 by the method of Edman (20) revealed an unblocked initiator methionine on 80% of the molecules (R. Hewick, personal communication). This is not found in the wild-type protein (21). T4 DNA ligase and radioisotopes were purchased from New England Nuclear. BAL-31 exonuclease was purchased from Bethesda Research Laboratories. Restriction enzymes were obtained from New England Biolabs.

For NMR study, the wild-type and mutant repressors were exhaustively dialyzed against 200 mM ammonium bicarbonate and lyophilized. The powder was dissolved in 99.7% $^2$H$_2$O, lyophilized, dissolved in 340 $\mu$L of NMR buffer (see below), lyophilized, dissolved in 340 $\mu$L 99.96% $^2$H$_2$O, centrifuged for 1 min in a microcentrifuge, and placed in a 5-mm NMR tube. The p$^3$H was adjusted with small aliquots of NMR buffer/50 mM KOH or NMR buffer/50 mM $^2$HCl. NMR buffer consists of 50 mM potassium phosphate (p$^3$H 7.4, direct electrode reading), 200 mM KCl, 1 mM sodium azide, and 0.1 mM EDTA in 99.96% $^2$H$_2$O. The protein con-

Fig. 2. Construction of plasmids that overproduce mutant repressors containing NH2-terminal deletions. (Lower Right) Plasmid pJE444 overproduces a mutant phage \( \lambda \) repressor with amino acids 4–236. It was constructed from two different DNA fragments. The first fragment was derived from pTAC2B (Upper Right), which provides a strong promoter, tac (16, 17), followed by a ribosome binding site (SD) and a translation initiation codon, ATG (C. Shoemaker, personal communication). These control functions (single-limed portion) lie between the Cla I and BamHI sites of pBR322 (open-box portion). pTAC2B was digested with Nco I; the sticky ends were made flush with Escherichia coli DNA polymerase I large fragment and the four dNTPs; the flush-ended linear plasmid was digested to completion with Nar I; the large fragment containing the promoter and origin of replication was isolated. The coding region was obtained from plasmid pKB252 (18), which was digested with HglAI, cutting at the beginning of the cl gene. The fragments were treated with exonuclease BAL-31 for 10–100 sec at 1 unit of enzyme per 100 

mu g of DNA; the time points were pooled and digested with Hpa II; the cl-containing fragments (heterogeneous at the BAL-31 end) were isolated. Finally, the promoter fragment and the coding fragment were ligated. The translation initiation codon ATG, generated by filling in the Nco I site, was joined to the various deletie end-points in the cl gene, and the Nar I sticky end was joined to the identical Hpa II sticky end, giving pJE444. pJE442, encoding a 2–236 phage \( \lambda \) repressor, was isolated from the same construction experiment as pJE444, pJE437, which encodes the 7–236 cl gene, was constructed similarly, but the cl-containing fragment came from a plasmid that differed after the 3' end of the cl gene. (Left) DNA sequence of pJE444 around the start of the cl gene, showing an in-frame deletion beginning with the fourth codon.

concentration was about 3 mM, assuming that a concentration of 1 mg/ml has an absorbance at 280 nm of 1.12 cm\(^{-1}\) (14).

NMR experiments were performed at 500 MHz and 30°C. The J-correlated spectrum (12, 13, §) was obtained by the method of States et al. (22), as described in the legend to Fig. 5.

GENERAL STRATEGY

In this section, we describe dynamic features of the two-dimensional correlated experiment. These features are intrinsic to the pulse sequence and are not dependent upon the molecule to be studied. In the next section, we illustrate the specific application of this experiment to phage \( \lambda \) repressor.

The pulse sequence of the correlated experiment is 90°-t\(_1\)-

90°-t\(_2\). After Fourier transformation with respect to \( t_1 \) and \( t_2 \), cross-peaks are observed which connect spins that are scalar-coupled (12, 13, §). The pattern of cross-peaks delineates spin systems (e.g., \( AX, AMX \ldots \)). Since the covalent structure of the peptide bond precludes scalar coupling between successive residues, each amino acid constitutes an independent spin system. In principle, the correlated spectrum of a protein is the sum of its constituent spin systems. This can be an important aid in classifying the 1H NMR resonances of small proteins, making possible a sequential assignment strategy (23–25). However, dynamic features of the experiment prevent the extension of this strategy to larger systems.

In the two-dimensional correlated experiment, cross-peak amplitude is sensitive to linewidth in two ways. First, since the decay time of the coherence is inversely proportional to linewidth, the period of effective signal accumulation (and thus total signal) decreases as \( T_2 \) (the transverse relaxation time) decreases. This is a general feature of all Fourier transform NMR experiments (including two-dimensional experiments). A second effect, which is a special feature of the correlated pulse sequence, results from the phase relationships in the fine structure of the cross-peaks. Each correlation signal appears as a cluster of distinct resonances with opposing phase and zero net integral (12, 13, §). The individual components of a cluster are separated by \( J \), the scalar-coupling constant. Since linewidths in the 1H NMR spectrum of macromolecules are often of magnitude comparable to \( J \), considerable phase cancellation may occur. Local flexibility, which can give rise to motional narrowing of NMR resonances (26–30), limits the extent of phase cancellation. Thus, the correlated experiment may be used to observe selectively flexible regions of macromolecules.

The relationship between linewidth and signal strength is shown in the lower panel of Fig. 3. For one-dimensional experiments, the relationship is linear (dashed line in Fig. 3). In two-dimensional Overhauser experiments, relaxation occurs during both time domains, and so the dependence is quadratic (dash/dot line in Fig. 3). Correlation signals, however, decay more rapidly as linewidths approach \( J \) (solid line in Fig. 3), resulting in nearly complete phase cancellation when linewidths exceed \( J \). This effect is illustrated by computer simulation in Fig. 4. In Fig. 4A, the linewidth is half the size of the coupling constant. This weakly-coupled AX coherence contains two positive and two negative peaks of equal size, with separation \( J \). In Fig. 4B, the linewidth is equal to the coupling constant. This increase in linewidth by a factor of 2 reduces cross-peak amplitude by 10-fold. Similar attenuation occurs in Fig. 4C, wherein the linewidth is twice \( J \).

APPLICATION

The correlated spectrum of phage \( \lambda \) repressor illustrates the effect of dynamic filtering. Repressor forms large oligomers at millimolar concentration whose molecular weight exceeds 1000 kDa (31, 32). The one-dimensional 1H NMR spectrum of these oligomers consists primarily of broad envelopes of resonances (32). In addition, there are a small number of sharp features (30). The correlated experiment can be used to observe and identify these features, since the many broad resonances undergo phase cancellation in two dimensions. The 236 residues of phage \( \lambda \) repressor constitute 236 spin systems. Each of these may in principle contribute several cross-peaks to the correlated spectrum. Thus, such a protein spectrum may contain hundreds of cross-peaks (22–24). The repressor spectrum, however, contains less than a dozen. The apparent simplicity of this spectrum is a result of dynamic filtering. The most prominent spin systems are those of serine and threonine. In addition, there are several lysines. The serine and threonine spin systems are shown in Fig. 5A. These may be assigned to the NH2-terminal residues serine-1 and threonine-2 by inspec-
The dynamics of proteins play an important role in ligand binding, catalysis, and macromolecular interactions (34, 35). For example, transient packing defects on a picosecond time scale may make possible oxygen uptake and release in myoglobin (36, 37). In addition to local fluctuations, global modes may occur (38). Hinge-bending in hexokinase and lysozyme (39–41), for example, accompanies binding of substrate to the active site cleft. Order–disorder transitions also represent an important dynamic feature. Their importance in protein–RNA interactions was suggested by Klug and co-workers in their studies of tobacco mosaic virus (42, 43). Similar features are observed in spherical RNA viruses, such as tomato bushy stunt virus (44, 45). Segmental flexibility may reduce kinetic barriers in viral assembly. This aspect of protein–RNA interactions also may occur in protein–DNA interactions (2, 11). A flexible NH2-terminal arm of phage λ repressor is thought to wrap around the double helix to contact the back of the operator site.

NMR spectroscopy is sensitive to both overall and local molecular motions and has been used to demonstrate segmental flexibility in proteins and viruses (27–29, 43, 49). Two-dimensional spectroscopy can facilitate such studies in several ways. By dispersing resonances in a second dimension, resolution is enhanced. In addition, relationships among spins are delineated, which is an important aid in their classification and assignment (13, 23–25). Finally, two-dimensional correlated spectroscopy (2D-COSY) multiplets contain second-order multiplet components, as shown in Fig. 3. Some linewidth aspects are illustrated in Fig. 4. The linewidth is determined by the second-order line-shape function of the spectral density function of the concerned motion, and thus, linewidth can be used to study the nature of the motion. For, example, linewidth and linewidth anisotropy have been used to test the concept of anisotropic tumbling for proteins (40). In the case of proteins, this tumbling model may be used to study the motions of flexible motifs or segments. Thus, the methodology of both two-dimensional NMR spectroscopy and two-dimensional linewidth analysis can be applied to study the motions of small proteins.

Fig. 3. NMR experiments differ in their sensitivity to linewidth and, thus, to the effects of motionally narrowing. In one-dimensional Fourier transform spectroscopy (dashed line), a linear dependence is observed. In two-dimensional spectroscopy, relaxation occurs in both dimensions, giving rise to a second-order dependence. Two-dimensional multiplet resonances depart from this second-order dependence as linewidths approach the coupling constant. In this regime, nuclear Overhauser effect spectroscopy (NOESY) multiplets (dash/dot line) coalesce and add constructively, resulting in a small increase in observed peak height. In contrast, correlated spectroscopy (COSY) multiplets (solid line) interfere destructively, diminishing the signal. This interference provides a dynamic filter, as illustrated in Fig. 4.

Fig. 4. Computer simulation showing the effects of linewidth in two-dimensional correlated spectroscopy. When the linewidth is less than the coupling constant J (A), the individual components of the two-dimensional multiplet are well resolved, giving rise to a strong signal. When the linewidth is comparable to J (B), phase cancellation occurs, diminishing the signal. For linewidths greater than J (C), almost complete cancellation results.

DISCUSSION

The resonances of serine-1 and threonine-2 would have been difficult to assign by conventional methods. Because repressor precipitates below neutral pH at these concentrations, serine-1 cannot be assigned by NH2-terminal titration. In addition, the exchange rates of amide protons in disordered regions are too fast to permit observation of their Overhauser effects.
dimensional signal strength may be exquisitely sensitive to motional narrowing.

The usefulness of dynamic filtering in macromolecular studies arises from a coincidence of time scales. The value of the scalar coupling constant $J$ is determined by the covalent structure of the amino acids. Although modulated to some extent by local dihedral angles (46), $J$ values fall in a characteristic range that is independent of molecular weight or rate of overall rotation. Thus, the nature of the amino acids determines (through $J$) the time scale for phase cancellation in two-dimensional correlated experiments. It happens that this is the same time scale that characterizes the tumbling of proteins. The rotational correlation time of lysozyme, for example, is about 8 nsec (47). Treating this single-domain protein as an isotropic rigid rotor, we may calculate a typical dipolar linewidth of 10 Hz at 500 MHz. This is also a typical value of $J$ for amino acids (48).

CONCLUSIONS

The NH$_2$-terminal arm of phage $\lambda$ repressor is shown to be flexible in solution by a new application of two-dimensional $^1$H NMR spectroscopy. The J-correlated experiment acts as a dynamic filter, permitting flexible regions to be identified. This effect may be of general utility in the study of large proteins and macromolecular assemblies.

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