Methyl Motions in ^{13}C-Methylated Concanavalin As Studied by ^{13}C Magnetic Resonance Relaxation Techniques†

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ABSTRACT: The carbon-13 spin–lattice relaxation times and nuclear Overhauser enhancements of the N^{ϵ}-monomethyllysine, $N^{\epsilon},N^{\epsilon}$-dimethyllysine, and N^{α},N^{α}-dimethylalanine resonances of ^{13}C-methylated concanavalin A have been measured at three carbon frequencies and compared to the relaxation parameters predicted by several motional models. The experimental parameters cannot be reproduced by a simple dipolar relaxation model which includes isotropic reorientation of the protein plus free internal rotational diffusion of the methyl groups but are well predicted by a wobble in a cone model which includes isotropic reorientation of the protein at 33 ns, free internal rotational diffusion of the methyl groups, and a wobble diffusion which reflects the net motion of the amino acid side chains. The analysis indicates that the methylated ϵ-amino side chains exhibit only slightly more motional freedom than does the methylated N-terminal α-amino group and suggests some restriction of methyl group rotation in the dimethylamino residues.

Concanavalin A (Con A)[1] is a metalloprotein isolated from the jack bean. The physical properties of this protein have been widely studied in recent years because of its ability to recognize and bind to specific membrane receptors on cellular surfaces. We have been exploring ways which may be used to examine these protein–cell interactions using high-resolution NMR techniques; one approach is to label surface residues on Con A with NMR-active nuclei which might be sensitive to interactions occurring between the protein and a cell surface. Stellwagen et al. (1977) converted the surface lysines of cytochrome *c* to homoarginine residues by using 90% [^{13}C]-*O*-methylisourea and showed that the resulting ^{13}C resonances were sensitive to the chemical environment and segmental motion experienced by each homoarginine residue. Biological methods have been successfully used to label dihydrofolate reductase with [^{13}C]arginine (Cocco et al., 1978). The resulting ^{13}C resonances were also found to be sensitive to conformational changes produced by the binding of substrate and inhibitor molecules to the enzyme and to motions experienced by these individual nuclei. More recently, Jentoft et al. (1981) reductively methylated ribonuclease A using 90% [^{13}C]formaldehyde and found that $N^{\epsilon},N^{\epsilon}$-dimethyllysine-41, an active-site residue, shows a unique methyl chemical shift that is sensitive to the presence of enzyme substrates and inhibitors. This unique resonance also has a shorter spin–lattice relaxation time (T_1) and a smaller nuclear Overhauser enhancement (NOE) than the remaining methylated lysine residues, indicating that these parameters are sensitive to the chemical environment of these methylated lysine residues.

We have previously reported that Con A may be reductively methylated to yield a derivative which retains its ability to bind with simple saccharides and agglutinate polysaccharides (Sherry & Teherani, 1983). We report here spin–lattice relaxation times and nuclear Overhauser enhancements for the three chemically distinct methylamine derivatives that are formed in this reaction and show that the relaxation parameters of these methyl groups are sensitive to the various motions experienced by these surface-labeled groups.

Materials and Methods

Protein Methods. Concanavalin A was prepared by the affinity chromatography method as described by Agrawal & Goldstein (1967), purified of its fragmented subunits as described by Cunningham et al. (1972), and finally treated with 1 M acetic acid to remove the native metal ions (Brown et al., 1977). The purified protein was reductively methylated by using 1–2 equiv of 90% [^{13}C]formaldehyde as previously outlined (Sherry & Teherani, 1983) to ensure partial formation of N^{ϵ}-monomethyllysines. The derivatized product was dialyzed extensively against pure water and against 0.3 M NaCl and 0.05 M sodium acetate, pH 5.6, and concentrated to 5–20 mg/mL for NMR relaxation experiments.

NMR Experiments. The carbon NMR experiments were performed at 15.1 MHz on a Bruker WP-60 spectrometer, at 25.2 MHz on a Varian XL-100-15 spectrometer equipped with a Nicolet TT-100 Fourier transform accessory, and at 50.1 MHz on a JEOL FX-200 spectrometer. Samples were maintained at a probe temperature of 25 ± 1 °C by using N_2 or dry air.

Spin–lattice relaxation times were determined by the inversion–recovery method, and nuclear Overhauser enhancements were measured by using gated proton decoupling techniques with a recycle time $\geq 5T_1$. Our estimated uncertainty in the reported T_1 and NOE values is ±10%. The computer calculations for the modeling were performed on a PDP-1170 at the University of California at San Francisco and on a PDP 11/45 at the University of Texas at Dallas.

Results

^{13}C Relaxation Measurements. The 50.1-MHz ^{13}C NMR spectra of partially methylated Con A prepared by using 90% [^{13}C]formaldehyde are shown in Figure 1. The procedures

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[1] Abbreviations: Con A, concanavalin A; MeCon A, methylated concanavalin A; SDS, sodium dodecyl sulfate; TNBS, trinitrobenzenesulfonate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement.

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Table I: Spin–Lattice Relaxation Rates and Nuclear Overhauser Enhancements at 50.1 MHz of Three Partially Methylated Concanavalin A Derivatives

	N^{ϵ}-monomethyllysine envelope (33.3 ppm)			$N^{\epsilon},N^{\epsilon}$-dimethyllysine envelope (43.1 ppm)			N^{α},N^{α}-dimethylalanine (41.8 ppm)		
reaction pH	no. of residues labeled	T_1 (s)	NOE	no. of residues labeled	T_1 (s)	NOE	no. of residues labeled	T_1 (s)	NOE
6.0	1.4	1.02	2.4	0.4	0.70	2.7	1.0	0.46	2.6
6.5	2.0	0.99	2.7	1.2	0.67	2.7	1.0	0.57	3.2
7.0	1.0	1.04	2.5	2.0	0.64	2.8	1.0	0.52	2.6
		1.02 ± 0.03[a]	2.5 ± 0.15[a]		0.67 ± 0.03[a]	2.73 ± 0.03[a]		0.52 ± 0.06[a]	2.8 ± 0.35[a]

[a] Average value.

for methylating the protein have been outlined previously (Sherry & Teherani, 1983). The large envelope of resonances centered at 43.1 ppm and the smaller sharp resonance at 41.8 ppm have been assigned (Sherry & Teherani, 1983) to $N^{\epsilon},N^{\epsilon}$-dimethyllysine and N-terminal N^{α},N^{α}-dimethylalanine resonances, respectively, on the basis of their chemical shift similarities to those resonances in ^{13}C-methylated ribonuclease, albumin, and lysozyme (Jentoft & Dearborn, 1979; Jentoft et al., 1979; Gerken et al., 1982) and their pH titration behavior (Sherry & Teherani, 1983). Similarly, the smaller envelope of resonances centered at 33.3 ppm may be assigned to N^{ϵ}-monomethyllysine residues since these resonances are absent in Con A products which have been completely dimethylated. The two broad resonances centered at 44.1 and 40.9 ppm have been tentatively assigned (Sherry & Teherani, 1983) to the magnetically nonequivalent methyl groups of $N^{\epsilon},N^{\epsilon}$-dimethyllysine-101 which forms a strong salt bridge with Asp-203 (Reeke et al., 1975).

The partially methylated Con A, whose spectrum is shown in Figure 1, contained 0.85 methyl group per protein amino group as assayed by ^{14}C and approximately 5 ± 1 free amino groups as assayed by TNBS. This information, together with the integrals obtained from the ^{13}C spectrum recorded in the absence of a NOE, indicates that this derivative contains an average of 5.6 dimethyl- and 1.5 monomethylamino groups per protein monomer. It seems probable that this partially methylated product is quite heterogeneous as evidenced by the hint of four or five overlapping resonances in the N^{ϵ}-monomethyllysine region. The following question then arises: Do each of the $N^{\epsilon},N^{\epsilon}$-dimethyl- and N^{ϵ}-monomethyllysine residues which contribute to the envelopes centered at 43.1 and 33.3 ppm exhibit similar relaxation behavior? The average chemical shift observed for each of these envelopes is identical with those observed in a SDS-denatured sample, indicating that these lysines are all surface residues and readily accessible to the solvent. Stellwagen et al. (1977) and Cocco et al. (1978) have observed relationships between the chemical shift and segmental motion (as measured by T_1's) of surface-exposed residues; i.e., a residue experiencing decreased motion will also be chemically shifted the furthest from the position of the solvent-exposed residues. We have been unable to detect any significant differences in the spin–lattice relaxation times of the shoulders of these two envelopes which suggests that all residues which comprise each envelope do indeed relax similarly.

To examine this question further, the spin–lattice relaxation times and nuclear Overhauser enhancements of the dimethyl- and monomethyllysine envelopes were determined on three different methylated Con A products which resulted from reaction of 1 equiv of $^{13}CH_2O$ at pH 6.0, 6.5, and 7.0. The three products had different numbers of both $N^{\epsilon},N^{\epsilon}$-dimethyllysine and N^{ϵ}-monomethyllysine residues as estimated from the resonance integrals, and yet the T_1's and NOE's of

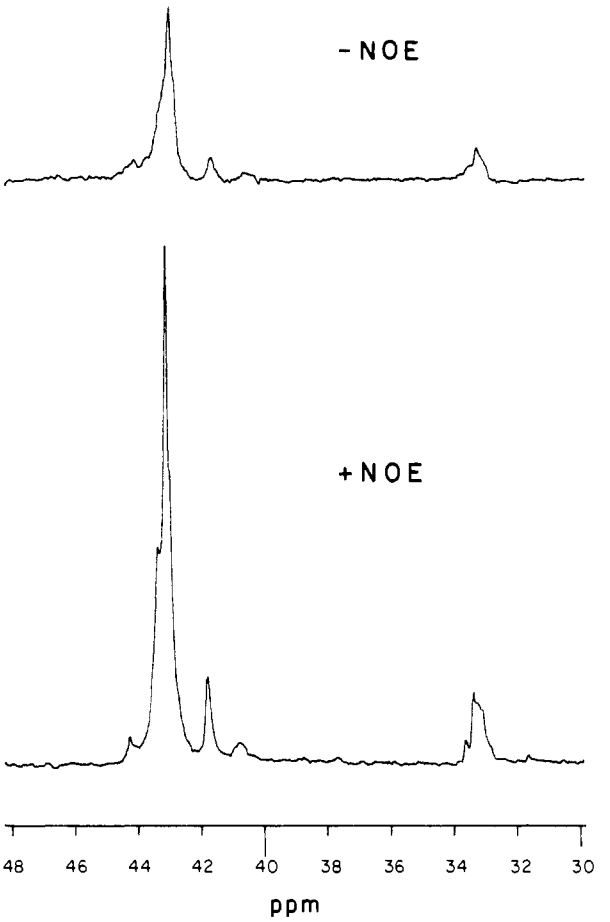

FIGURE 1: ^{13}C NMR spectra at 50.1 MHz on a 7.5 mg/mL sample of partially methylated Con A illustrating the $^{13}C\{^1H\}$ nuclear Overhauser effect. Each spectrum represents 4800 accumulations with an acquisition time of 0.51 s and a delay time of 6 s. The chemical shifts are reported with respect to internal methanol at 49.4 ppm.

their respective envelopes in each sample were identical to within ±6% (Table I). Furthermore, the $N^{\epsilon},N^{\epsilon}$-dimethyllysine envelope is resolved into four resonances at pH 8, one of which is a single lysine residue (Sherry & Teherani, 1983). The T_1's of these four resolved resonances at 50.1 MHz measured 0.71, 0.72, 0.72, and 0.61 s. If one generates four separate exponential curves based upon these T_1 values and averages the calculated magnetizations at each τ value, the resulting averaged curve (representing the envelope) decays exponentially with an effective T_1 of 0.687 s. We therefore conclude that the relaxation behavior of each individual lysine residue which comprises each envelope is the same within experimental error and the relaxation behavior of each envelope in the spectra at pH 5.6 should accurately reflect the average dynamic behavior of these surface-exposed residues.

The experimental spin–lattice relaxation times and nuclear Overhauser enhancements of partially methylated apo-Con

Table II: Relaxation Data for ^{13}C-Methylated Concanavalin A at Three Carbon Frequencies[a]

carbon frequency (MHz)	N^{ϵ}-monomethyllysine envelope (33.3 ppm)		$N^{\epsilon},N^{\epsilon}$-dimethyllysine envelope (43.1 ppm)		N^{α},N^{α}-dimethylalanine (41.8 ppm)	
	T_1 (s)	NOE	T_1 (s)	NOE	T_1 (s)	NOE
15.1	0.5 ± 0.1	1.3	0.5 ± 0.1	1.7	[b]	[b]
25.2	0.95	1.8 ± 0.1	0.55	2.1 ± 0.1	0.46	2.0 ± 0.1
50.1	1.02	2.5 ± 0.1	0.67	2.7 ± 0.1	0.52	2.8 ● 0.1

[a] The experimental error in the T_1's and NOE's is estimated at ±10% unless otherwise indicated. [b] Poor signal to noise ratios precluded a measurement of these values.

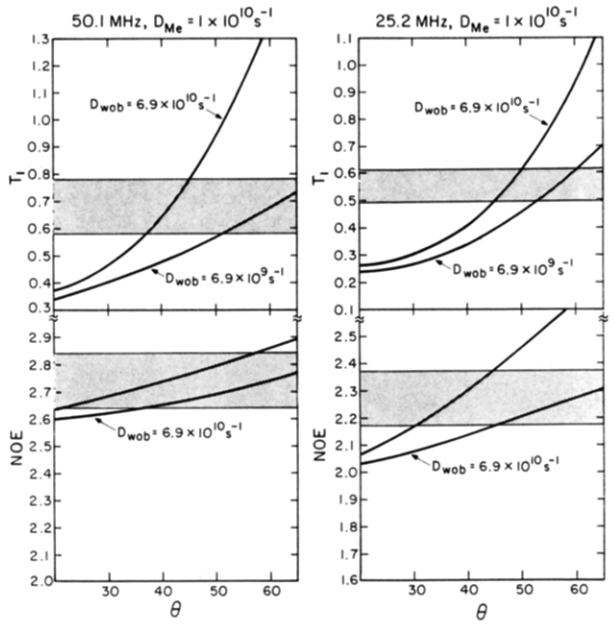

FIGURE 2: Calculated ^{13}C T_1 and NOE curves using the wobble in a cone model for $N^{\epsilon},N^{\epsilon}$-dimethyllysine as a function of the wobble half-angle (θ). Curves are shown for D_{wob} = 6.9 × 10^9 and 6.9 × 10^{10} s^{-1} while D_{Me} was held constant at 1 × 10^{10} s^{-1}. The solid horizontal areas represent the experimentally allowed T_1 and NOE values for the $N^{\epsilon},N^{\epsilon}$-dimethyllysine envelope of resonances determined at the two indicated carbon frequencies.

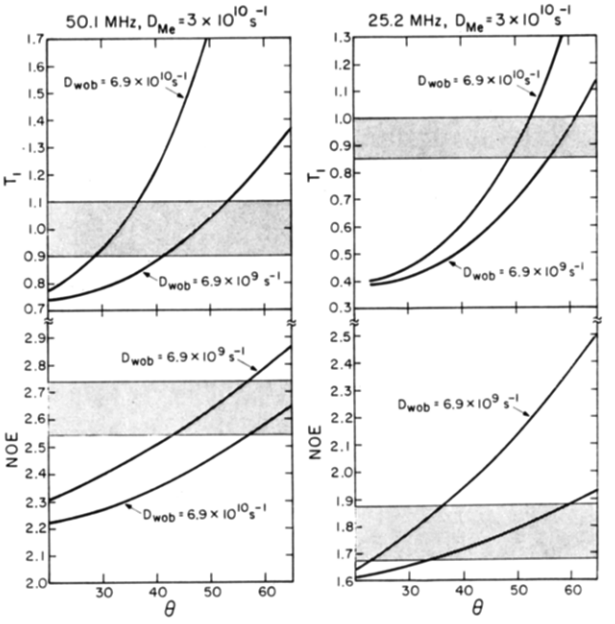

FIGURE 3: Calculated ^{13}C T_1 and NOE curves using the wobble in a cone model for N^{ϵ}-monomethyllysine as a function of the wobble half-angle (θ). The curves shown were determined for D_{Me} = 3 × 10^{10} s^{-1}.

A at three carbon frequencies are summarized in Table II. The relaxation parameters were independent of protein concentration up to 50 mg/mL, added diamagnetic Zn^{2+} and Ca^{2+} ions, and added methyl α-D-mannopyranoside.

Motional Modeling. To gain some insight into the molecular motions of the ^{13}C-enriched methyl groups on the surface of Con A, T_1 and NOE values were calculated for several models by using standard expressions given elsewhere (Doddrell et al., 1972; James et al., 1978; Wittebort & Szabo, 1978; London & Avitable, 1978; Richarz et al., 1980; Brainard & Szabo, 1981). In all models, ^{13}C relaxation was assumed to be dominated by dipolar interactions with three directly bonded protons at a distance of 1.11 Å. An overall correlation time of 33 ns for dimeric Con A was estimated from the ^{13}C α-carbon backbone T_1 value obtained at 50.1 MHz by using an isotropic protein tumbling model. This value agrees favorably with the value of 31 ns determined for dimeric succinyl-Con A from fluorescence lifetime data (Yang et al., 1974).

The specific models examined were similar to those chosen for describing the [^{13}C]methyl relaxation rates of methionines in myoglobin (Wittebort & Szabo, 1978) and in dihydrofolate reductase (Blakley et al., 1978). A simple model which assumes rapid methyl rotation as the only internal motion gave poor agreement between calculated and measured T_1 and NOE values for all three resonances at each frequency over a wide range of methyl diffusion constants (10^9–10^{12} s^{-1}), where the diffusion constant is related to the correlation time describing the motion by $(6\tau_c)^{-1}$. In each case, the experimental T_1's and NOE's were larger than the calculated values, indicating additional internal motions contribute to the relaxation behavior of all three types of methylated residues.

Next, a calculation was performed which allowed two free diffusion internal rotations in addition to the overall isotropic motion of the protein. D_{Me} was varied from 1 × 10^{10} to 5 × 10^{10} s^{-1}, typical for methyl groups (Blakley et al., 1978; Richarz et al., 1980; Wittebort et al., 1979; Jones et al., 1976; Inagaki et al., 1982), and D_{Int}, representing free diffusion about the C^{ϵ}–N^{ϵ} or C^{α}–N^{α} bonds, was varied from 10^9 to 10^{12} s^{-1}. Reasonable agreement was found between calculated and experimental relaxation parameters at 50.1 MHz for $N^{\epsilon},N^{\epsilon}$-dimethyllysine and N^{α},N^{α}-dimethylalanine when D_{Me} = 5 × 10^{10} s^{-1} and D_{Int} = (3–5) × 10^9 s^{-1}, but poor agreement was found for the corresponding 25.2- and 15.1-MHz relaxation parameters. For the latter two frequencies, the calculated NOE's were significantly larger than the experimental values over these same internal diffusion ranges. The N^{ϵ}-monomethyllysine data at any of the carbon frequencies could not be fit with this model.

Finally, calculations were performed by using a wobble in a cone model as used by Brainard & Szabo (1981) for describing several aliphatic side chain methyl motions in the basic pancreatic trypsin inhibitor protein. Our model assumed that the combined motions of the lysine side chain $-CH_2-$ groups or the N-terminal alanine peptide backbone could be described by a net wobbling motion of the N–CH_3 bond through ±θ°.

Table III: Calculated Relaxation Data for a Wobble in a Cone Motional Model

resonance	carbon frequency (MHz)	θ (deg)	allowable diffusion constants		calcd	
			D_{wob} (s^{-1})	D_{Me} (s^{-1})	T_1 (s)	NOE
N^{ϵ}-monomethyllysine	15.1	50	$(0.69–6.9) \times 10^{10}$	$(1.2–10) \times 10^{10}$	0.52	1.30
	25.2	50	$(0.69–6.9) \times 10^{10}$	$(1.2–10) \times 10^{10}$	0.95	1.77
	50.1	50	$(0.69–6.9) \times 10^{10}$	$(1.2–10) \times 10^{10}$	1.02	2.64
$N^{\epsilon},N^{\epsilon}$-dimethyllysine	15.1	50	$(0.69–6.9) \times 10^{10}$	$(0.6–2.9) \times 10^{10}$	0.40	1.70
	25.2	50	$(0.69–6.9) \times 10^{10}$	$(0.6–2.9) \times 10^{10}$	0.55	2.27
	50.1	50	$(0.69–6.9) \times 10^{10}$	$(0.6–2.9) \times 10^{10}$	0.68	2.74
N^{α},N^{α}-dimethylalanine	15.1	40	$(0.9–18) \times 10^{9}$	$(0.9–10) \times 10^{10}$	0.32	1.33
	25.2	40	$(0.9–18) \times 10^{9}$	$(0.9–10) \times 10^{10}$	0.46	2.00
	50.1	40	$(0.9–18) \times 10^{9}$	$(0.9–10) \times 10^{10}$	0.52	2.69

Free unrestricted diffusion of the methyl group about this bond (D_{Me}) was varied from 10^9 to 10^{12} s^{-1}, D_{wob} was varied from 5×10^8 to 5×10^{11} s^{-1}, and the wobble half-angle (θ) was examined from 20° to 110°. Figures 2 and 3 illustrate the sensitivity of the calculated T_1 and NOE values to θ and D_{wob} for the $N^{\epsilon},N^{\epsilon}$-dimethyllysine and N^{ϵ}-monomethyllysine data, respectively. The shaded area in each figure represents the experimental T_1 and NOE error limits for data at each frequency. Both T_1 and NOE are insensitive to D_{wob} at small wobble half-angles but become very dependent upon this parameter at larger values of θ. At any given wobble diffusion constant, both calculated T_1 and NOE values increase with increasing θ. Conversely, at any given wobble angle, the calculated T_1's increase and NOE's decrease with increasing D_{wob}. The net result is that rather narrow limits are placed upon D_{wob} and θ at a given methyl rotational diffusion constant. This is illustrated by the calculated curves in Figure 2 for $D_{Me} = 1 \times 10^{10}$ s^{-1}. First, consider the 50.1-MHz data. The $D_{wob} = 6.9 \times 10^{10}$ s^{-1} curves indicate that acceptable values for T_1 and NOE are found between $\theta = 37°$ and $\theta = 46°$ while the corresponding $D_{wob} = 6.9 \times 10^9$ s^{-1} curves limit θ to 52–57°. If two new curves were generated between these upper and lower D_{wob} limits representing $D_{wob} = (1 - 3) \times 10^{10}$ s^{-1}, θ would be limited to 50 ± 8°. An inspection of the 25.2-MHz data indicates that θ is limited to 52 ± 5° for this same D_{wob} range.

It is important to note that a different value for D_{Me} generates a different acceptable D_{wob} range for this same $N^{\epsilon},N^{\epsilon}$-dimethyllysine data but the limits on θ remain near 50 ± 10°. Like D_{wob}, limits on D_{Me} are also imposed by the opposite sensitivity of T_1 and NOE values to this diffusional motion. The somewhat smaller T_1 and NOE values measured for the unique N^{α},N^{α}-dimethylalanine resonance shift the allowable values of θ to 40 ± 10°. Limits on the range of D_{wob} and D_{Me} motions are summarized in Table III.

Although the N^{ϵ}-monomethyllysine data shown in Figure 3 can also be fit over a narrow range of D_{wob} and θ values, the overall fit of these data to the wobble model is less satisfactory. At $D_{Me} = 3 \times 10^{10}$ s^{-1}, the 25.2-MHz data fit best at higher D_{wob} constants while the 50.1-MHz data fit best at lower D_{wob} constants. Similarly, at any given D_{wob} value, the 50.1-MHz data predict higher D_{Me} constants than do the 25.2-MHz data. The D_{wob} and D_{Me} ranges reported in Table III for the N^{ϵ}-monomethyllysine data are limited at the upper and lower boundaries by the 50.1- and 15.1-MHz data, respectively. In this case, only values near $D_{Me} = 3 \times 10^{10}$ s^{-1}, $D_{wob} = 2 \times 10^{10}$ s^{-1}, and $\theta = 50°$ predict T_1 and NOE values within the experimental limits at the three carbon frequencies.

Discussion

The spin–lattice relaxation rates and nuclear Overhauser enhancements of the ^{13}C-enriched methyl groups chemically introduced into the tertiary structure of Con A are found to be quite sensitive to the motions experienced by these surface-exposed groups. A simple dipolar relaxation model which assumes isotropic rotation of dimeric MeCon A at 33 ns plus free internal diffusive rotation about the methyl axes cannot reproduce the experimental relaxation parameters within their allowable error limits. Introduction of a second free internal diffusive rotation about the C^{ϵ}–N^{ϵ} or C^{α}–N^{α} bonds improves the fit dramatically for the dimethylamine data at 50.1 MHz but not at the lower carbon frequencies. If our experiments had been run at this single frequency, it would have been possible to fit the dimethylamine data with this model, but we may have erroneously concluded that the monomethyllysines experience considerably different motions than do the dimethyllysines.

The relaxation data for all three types of methyl resonances at each frequency can be fit by a wobble in a cone model which restricts the motion of the N–CH_3 vector through ±θ°. Table III summarizes the allowable values of D_{wob} and D_{Me} which reproduce the experimental T_1's and NOE's. In each case, the highest value of D_{Me} and the lowest value of D_{wob} gave the indicated results as did the reverse combination, i.e., the lowest value of D_{Me} and highest value of D_{wob}. It is interesting to note that the allowable ranges for D_{wob} are identical for the monomethyl- and dimethyllysine resonances and these values are in general larger than the range of D_{wob} values found for the N-terminal dimethylalanine resonance. This suggests there is greater flexibility in the lateral movement of the N^{ϵ}-methyl groups than in the N^{α}-methyl group. Furthermore, if one assumes that D_{wob} is the same for the monomethyl- and dimethyllysine resonances, then D_{Me} must be consistently larger for the monomethyllysines to reproduce the experimental results, indicating that these methyl groups rotate more rapidly than those in the doubly labeled amines, perhaps due to steric interactions between the two methyl groups in the latter case. The range of D_{Me} frequencies given in Table III is similar to those determined for other protein side chain methyl groups from NMR data using the wobble in a cone or restricted diffusion models (Blakley et al., 1978; Wittebort et al., 1979; Richarz et al., 1980; Inagaki et al., 1982).

The calculated curves in Figures 2 and 3 show that the relaxation parameters are very sensitive to the wobble half-angle (θ). The limits for this angle were restricted to be near 50° for the monomethyl- and dimethyllysine data but were consistently found about 10° smaller for the N-terminal dimethylalanine resonance, which again suggests that this α-amino group experiences less motional freedom than the ϵ-amino groups. The values of θ determined in this study are similar to those determined for several methyl side chain residues in bovine pancreatic trypsin inhibitor (Richarz et al., 1980) by using a similar model.

We have not included line widths in our motional analysis

for the following two reasons: (1) there are multiple overlapping resonances in the monomethyl- and dimethyllysine envelopes; and (2) the dimethylamino resonances are exchange broadened at this pH due to slow inversion of the protonation amine (Goux et al., 1984) which exchanges the two diastereotopic methyl groups on the amine. Many of these limitations are removed when Co^{2+} and Ca^{2+} are added and the resulting metalloprotein is titrated to pH 8 or higher (Sherry & Teherani, 1983), but a relaxation rate analysis of the resolved resonances at high pH is tenuous because of possible paramagnetic metal ion contamination. Nevertheless, the wobble model parameters determined in this study predict methyl resonance line widths of 4–4.5 Hz, and these are very close to those observed in the resolved spectra of Co^{2+}–Ca^{2+}–MeCon A at high pH (Sherry & Teherani, 1983). This allows us to predict the line widths of these resonances when MeCon A is bound to cell surfaces. Inbar et al. (1973) have estimated from fluorescence data that the overall rotational correlation time for tetrameric Con A increases from 58 ns to 70–120 ns when the protein is bound to surfaces of lymphocytes or fibroblasts. If we assume that D_{Me} and D_{wob} are not altered for those methyl groups which do not come into direct contact with the cell surface, then their ^{13}C line widths at 50.1 MHz are predicted to fall between 7 and 14 Hz. This suggests that it might be possible to resolve several individual methyl resonances that are paramagnetically shifted away from their diamagnetic positions in Co^{2+}–Ca^{2+}–MeCon A while this protein is bound to cell surfaces. Such studies are in progress in our laboratories.

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