Resolved Librational Motions of Gramicidin-S and Glutathione Dimer as studied by Nuclear Magnetic Resonance Spectroscopy

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A theory relating ^{13}C n.m.r. relaxation parameters to molecular motion at three levels is described, and successfully subjected to unusually rigorous experimental test. The results give information on mean conformations and on rates of internal librational motions for gramicidin-S and for glutathione dimer. They also confirm some previous conclusions regarding proteins, although in this case the model is more approximate.

THERE has been considerable progress recently in the analysis of the motions of complex molecules by ^{13}C n.m.r. relaxation and nuclear Overhauser enhancement measurements. London and Avitabile [1] have presented a general theory of the relaxation parameters for atoms in a side-chain with freely rotating links attached to a rigid core, and although this model does not give very satisfactory fits with experiment,[2] its general approach may readily be developed to allow for restricted rotation of the links,[3] and other comparable motions,[4] in a way that has been shown to fit a few experiments quite satisfactorily. Howarth [5,6] has developed a generalised version of this theory which reinterprets the restricted rotations as internal librations specified only by their average angular extent and rate and which is thus applicable to molecular loops such as in proline and in the main chains of proteins as well as to side-chains. Jardetzky [7] has also recently proposed a more abstract analysis of the same problem.

In order to explain observations on natural rubber, Howarth [8] found it necessary to assume both slow, wider and fast, small angle librations superimposed on a very slow pseudo-isotropic motion of the polyisoprene chain. The relatively tractable theoretical model that was developed for this analysis involves five motional parameters, which is about as many as one might hope to determine reliably by currently achievable experiments. The aim of the present work is to give this model as rigorous an experimental testing as reasonably possible, and thus to go on to a more detailed experimental analysis of molecular flexibility than has hitherto been possible.

Accordingly, we have tested the theory on two medium-sized molecules, gramicidin-S and glutathione dimer, for which T_1 data are available or measurable for at least three observation frequencies, together with nuclear Overhauser enhancements (n.O.e.s) at some frequencies. In the case of glutathione dimer the experiments were conducted in 50% glycerol–^2H_2O, with the aim of slowing the larger angle motions preferentially and thus improving the resolution of the theoretical analysis into underlying components of motion. Linewidths were not included in the analysis because they can often be misleadingly increased by conformational changes on the millisecond timescale.

Most of the data on these compounds cannot be convincingly reproduced by a single-libration model, but the present model fits all the data within experimental error, and even passes such severe tests as giving the same underlying overall molecular rotation rate from data for different atoms in the same molecule even when this is not assumed in advance. The one failure found so far is that the double-libration model still overestimates some n.O.e.s in proteins, albeit not as badly as does the single-libration model.

EXPERIMENTAL

T_1 Measurements were carried out at 22.6 MHz and T_1 and n.O.e. measurements at 45.2 and 100.63 MHz on Bruker WH 90, WH 180WB, and WH 400 spectrometers, respectively, at room temperature. Decoupler heating was reduced on the WH 400 spectrometer by switching of power levels and alternation of decoupler sequences during n.O.e. measurement. Measurements were repeated, with T_1s having a precision of $\pm 10\%$ and n.O.e.s of $\pm 20\%$.

Gramicidin-S was obtained from Sigma and was used without further purification at a concentration of 0.088M in [$^2H_4$]methanol.

Glutathione dimer was prepared by the method of Rall and Lehringer,[9] and was used at 0.16M in 50% glycerol–^2H_2O for reasons described above. T_1 and n.O.e. values were assumed to be dominated by dipolar relaxation processes [10] and T_1 measurements were repeated twice.

THEORETICAL

The earlier, single-libration model [5,6] was developed from the single-rotor side-chain calculations of Woessner.[10] Its extension to double libration [6] was heuristic. However, it may be extended more rigorously by applying the same manipulations to London and Avitabile's [1] multiple-rotor model, reduced to the two-rotor case. In this way the nine components of their asymmetric matrix each become integrals involving the libration semi-angles. The resulting equations were used for the analysis described below, although their predictions are in fact experimentally indistinguishable from the simpler, heuristic theory. The underlying libration, which corresponds to the first rotation out from the rigid core in London and Avitabile's model, is assumed in all cases to be the slower one. Its jump correlation time is τ_S and its mean semi-angle is θ. The corresponding parameters for the second, faster libration are τ_G and X, and all these are assumed to be superimposed on an overall isotropic rotation of diffusional correlation time τ_R.

Experimental T_1 values were fitted by a least-squares programme developed from a method published by Moore.[11]

As this minimises the function $\chi^2 = [T_1(\text{expt.}) - T_1(\text{calc.})]^2/T_1(\text{expt.})$, it in effect favours the higher-field measurements, with their longer values of T_1. N.O.e.s were not fitted for the peptides, as they are frequently less sensitive to the details of the underlying motion, but they were valuable as a check against false minima, and are recorded in the Tables. The methyl group T_1 values were not considered to be experimentally reliable, because there is some suspicion of non-exponential relaxation decay: also, they would test a different theory.

Some of the calculated parameters are less sensitive to the experimental data than others. With the data and theory used, we estimate that the error limits on τ_R are $\pm 10\%$, on τ_S $\pm 10\%$, on τ_G $\pm 14\%$, on θ $\pm 8\%$, and on X $\pm 8\%$.

The overall correlation time for isotropic tumbling, τ_R, was at first allowed to vary freely for several resonances, as a test of the method of analysis. The calculated values of τ_R were found to lie within $\pm 10\%$ of a mean value, which was then fixed as the τ_R for all resonances. Any effects of anisotropic motion are likely to appear as an apparent contribution to the slow libration. We found that if the guessed starting value of τ_R was more than *ca.* 25% too small, new minima were found in some cases. These false minima could be reliably excluded because with the incorrect figures, $\Sigma\chi^2$ for all resonances was significantly larger both for values of T_1 and, independently, for values of the n.O.e.

Our method contrasts with that of Jardetzky,[7] who treats measurements at different fields independently. His resulting calculated motional parameters do not invariably harmonise, possibly because of T_2 errors, although they are generally consistent with our results.

RESULTS

The T_1 values for gramicidin-S in methanol at 15.2 and 67.5 MHz were taken from the work of Allerhand and Komoroski [12] and Komoroski *et al.*[13] The other results were obtained in the present study. The gramacidin-S results are in Table 1 and the glutathione dimer results in Table 2. Almost all the calculated T_1 values lie within 10% of the experimental ones, *i.e.* within experimental error. The calculated values of n.O.e also all lie well within the experimental error of 20%. Thus the two-libration model gives a very satisfactory fit with one of the largest available sets of experimental data.

Gramicidin-S.—On the present analysis α-carbons show no large-angle libration, and a rapid libration of very similar mean half-range X (0.47—0.51 rad) to that found in less tightly structured main chains (typically 0.5 rad). Both these observations are entirely consistent with the known structure of gramicidin-S [12] in methanol, which is a cyclic decapeptide with four cross-ring hydrogen bonds. They confirm that the rapid $X = 0.5$ rad libration is a localised α-carbon libration common to many different molecules and probably not involving the entire side-chain. τ_G, the rapid jump correlation time, is 97—109 ps. This is somewhat longer than the 60 ps deduced for proteins on a single-libration model, but the difference may not be significant because of the change of models. However, the slightly faster calculated motion of Orn α-C and especially of Phe α-C may be real, because only the Orn NH and Phe NH groups remain unrestricted by cross-ring hydrogen bonding. As no apparent or real slow librational motion is detected, we may assume that the overall tumbling of gramicidin-S is effectively isotropic and that the ring does not undergo major vibrational deformations, at least at room temperature.

In contrast, the side-chain motions all have a slow, wide-angle component of libration as well as a rapid one comparable with that found for the α-carbons. These slower librations have τ_S (typically) 300 ps, so that they are not

TABLE 1

Gramicidin-S: nT_1 values for different resonances, motional frequencies, and amplitudes from ^{13}C relaxation data

	Resonance frequency										Motion amplitude/frequencies[f]				
	15.2 MHz[b]		22.6 MHz[c]		67.5 MHz[d]		100.6 MHz[c]								
α-CH	nT_1[a] (obs.)	nT_1 (calc.)	nT_1 (obs.)	nT_1 (calc.)	nT_1 (obs.)	nT_1 (calc.)	nT_1 (obs.)	nT_1 (calc.)	n.O.e. (obs.)	n.O.e. (calc.)	τ_G/ps	X/rad	τ_S/ps	θ/rad	τ_R/ns
Pro-α	150	155	150	158	204	200	243	243	2.19	2.28	88	0.310	Very large	Very small	0.362
Val-α	144	154	162	157	210	199	242	242	2.11	2.28	92	0.305	Very large	Very small	0.362
Phe-α	141	160	150	163	203	206	250	250	2.16	2.29	80	0.347	Very large	Very small	0.362
Orn-α	135	159	158	162	177	204	248	248	2.11	2.29	90	0.342	Very large	Very small	0.362
Leu-α	147	147	*e*	150	201	191	232	232	2.09	2.27	95	0.234	Very large	Very small	0.362
Side-chain CH_2															
Orn-β	*g*	206	210	210	244	255	270	301	2.46	2.40	20	0.400	714	0.65	0.362
Orn-γ	*g*	369	375	375	448	450	540	520	2.23	2.48	20	0.550	96	0.70	0.362
Orn-δ	393	388	400	400	496	479	550	552	2.28	2.49	20	0.550	85	0.73	0.362
Leu-β	340	336	343	343	424	423	480	500	2.67	2.39	20	0.400	41	0.70	0.362
Phe-β	198	198	200	200	240	244	280	291	2.00	2.37	20	0.400	2 000	0.98	0.362
Side-chain CH															
Val-β	180	206	210	210	241	254	300	300	2.39	2.41	20	0.390	700	0.67	0.362
Leu-γ	421	402	412	412	511	510	635	605	2.67	2.37	20	0.500	28	0.71	0.362
Phenylalanine CH															
Phe δ	213	177	180	180	235	219	265	261	2.02	2.39	20	0.230	600	0.60	0.362
Phe ε	211	197	200	200	250	243	290	288	2.09	2.40	20	0.350	570	0.60	0.362
Phe ζ	174	174	*g*	177	196	216	275	256	2.46	2.39	20	0.210	692	0.63	0.362
Proline CH_2															
Pro-β	*g*	236	240	246	308	292	330	331	2.23	2.51	20	0.400	360	0.70	0.362
Pro-γ	*g*	291	*g*	295	344	344	380	391	2.09	2.59	20	0.410	211	0.79	0.362
Pro-δ	150	198	*e*	201	244	244	270	288	*e*	2.43	20	0.340	540	0.62	0.362

[a] nT_1 Values in ms. [b] Results from ref. 12. [c] Results obtained from this work at room temperature; concentration of 100 mg ml^{-1} in CD_3OD. [d] Results from ref. 13. [e] Accurate result could not be obtained because of interference of CD_3OD resonance. [f] τ_G = librational correlation time, X = mean librational angle (small-angle libration), τ_S = internal rotation or second librational correlation time, θ = second librational angle (large-angle libration), τ_R = overall reorientation correlation time. [g] Not available.

TABLE 2

Glutathione dimer [d]: nT_1 values for different resonances, motional frequencies, and amplitudes from ^{13}C relaxation data

	Resonance frequency										Motion amplitude/frequencies [e]				
	22.6 MHz		45.2 MHz				100.6 MHz								
	nT_1 [a] (obs.)	nT_1 (calc.)	nT_1 (obs.)	nT_1 (calc.)	n.O.e. [b] (obs.)	n.O.e. (calc.)	nT_1 (obs.)	nT_1 (calc.)	n.O.e. (obs.)	n.O.e. (calc.)	τ_G/ps	X/rad	τ_S/ps	θ/rad	τ_R/ns
Cys-α	70	67	105	111	1.63	2.01	214	224	1.84	1.59	70	0.300	891	0.90	6.0
Cys-β	83	80	146	124	1.83	2.17	220	235	1.50	1.50	70	0.350	711	0.95	6.0
Glu-α	119	132	175	175	2.22	2.48	283	287	2.07	2.02	20	0.470	500	1.13	6.0
Glu-β	149	155	220	229	2.10	2.37	377	366	1.97	2.32	20	0.500	300	1.00	6.0
Glu-γ	145	170	240	249	2.22	2.05	385	398	2.03	2.02	20	0.560	304	1.00	6.0
Gly-α	171	185	266	279	2.13	2.05	419	470	1.93	1.97	20	0.560	247	1.00	6.0

[a] nT_1 values in ms. [b] n.O.e. values at 45.2 and 100.63 MHz were obtained from the intensity ratio between a gated decoupled spectrum and a normal decoupled spectrum. [c] See footnote *f* of Table 1. [d] Relaxation data at room temperature; sample concentration of 0.16M in 50% glycerol-^2H_2O; pH 6.4.

much faster than the molecular rotation in this molecule. It is convenient to consider each side-chain in turn.

The ornithine side-chain shows fairly typical motional characteristics for a linear chain. The β-carbon is rather restricted in its motion, as predicted by Jones *et al.* on the basis of rotamer populations.[14] The γ- and δ-carbons show more motion, and with the δ-carbon this is on average more rapid, although not more extensive, in that a greater proportion of the motion is attributable to the rapid libration. One notable and unexpected effect of this is that the n.O.e. does not vary significantly along the chain. The ornithine side-chain appears on the above analysis to be flexible but boxed.

In contrast the phenylalanine side-chain is inflexible and boxed. The terminal ζ-carbon group has the smallest value of X in the entire molecule, presumably because the angle of the ζ-*C*–H bond is not affected by rotation about the β-C–γ-C bond. The T_1 differences between Phe δ-C and ε-C are probably experimentally insignificant and therefore so too are the calculated differences in librational parameters. The side-chain moves as a whole, with a rate typical for limited flexure at an α-C–β-C linkage,[5] plus some β-C–γ-C rocking.

The valine side-chain appears to be almost as motionally restricted as the ornithine side-chain at β-C. But in contrast, the leucine side-chain seems to be more flexible. It moves with about twice the angular extent and rate, consistent with the rotamer-population study.[14] This study revealed distinct preferences for single conformations of D-phenylalanine and ornithine but more extensive averaging of valine and leucine residues.

Finally, the proline ring shows motions consistent with London's detailed analysis.[15] As usual, the values of T_1 are in the order γ-C > β-C > δ-C. Our calculated variation in τ_S does, however, call into question the above authors' assumption of a single process of conformational change for the entire proline ring. It would instead seem that the Pro γ-C motion is to some extent a combination of semi-independent angular motions of β-C and δ-C.

Glutathione Dimer.—The data in Table 2 show that once again all the carbons have a fast component of motion whose semi-amplitude is *ca.* 0.5 rad. However, the motion of Cys β-C and particularly of Cys α-C is somewhat restricted, because of the S–S bridge. Also that of Gly α-C has X 0.56 which is consistent with earlier observations.[5] Evidently a side-chain partially restricts the rapid, local motions of the α-carbon of an amino-acid.

In contrast, the slow librations in glutathione dimer have a novel feature. Their maximum semi-amplitude θ is almost constant (0.96—1.07 rad) for every protonated carbon in the molecule, rather than increasing out from the molecular centre. This immediately shows that the four peptide chains extending from the Cys α-C atoms are not moving like flexible, largely unrestricted side-chains. The same conclusion is evident from the observation that one τ_R fits the entire molecule. Instead, all the slow librational motions appear to be confined within the same angular limits. One explanation of this is that the dimer molecule is cross-linked not only by the S–S bond but also by hydrogen bonds. Electrostatic interactions between the charged terminal groups may also play a limited role, although the conformation is apparently unaffected by added salt. The S–S and hydrogen bonds have a substantial but finite angular flexibility, which determines θ. Within these constraints, some atoms move more rapidly than others, particularly if they are not dynamically constrained by side-chains and associated solvent. Hence τ_S varies from 891 to 247 ps between Cys α-C and Gly α-C. Evidently the motional characteristics of a peptide molecular loop with loose links differ from those of a relatively stiff ring such as that of gramicidin-S, where no corresponding slow libration is detected.

We have studied the ^1H n.m.r. spectrum of the dimer in an attempt to identify these angular constraints more precisely. Unfortunately, anionic glutathione dimer is not readily soluble in dimethyl sulphoxide, and the solution that can be made gives the broad resonances characteristic of an aggregated product probably because of the molecular charge. This means that the standard method of identifying internal hydrogen bonds by the solvent effects on NH and CO resonances cannot be applied.

We have, however, compared the ^1H n.m.r. spectra of glutathione monomer (SH) at neutral pH in water with that of the dimer (S–S). The monomer shows no chemical shift inequivalence of the Gly α-protons, and only 0.050 p.p.m. for the Cys β-protons, whereas the dimer shows a difference of 0.054 for Gly α-H and 0.342 p.p.m. for Cys β-H. Also the coupling constants between Cys α-H and Cys β-H are 5.31 and 6.31 Hz in the monomer, but 4.41 and 9.58 Hz in the dimer. These observations are both consistent with the dimer being more conformationally restricted. For example, the approximate dihedral angles deduced from the Cys coupling constants *via* the Karplus equation, *ca.* 50 and 170°,* are consistent with the proposed cross-linked structure, but inconsistent with an open-chain, conformationally averaged molecule.

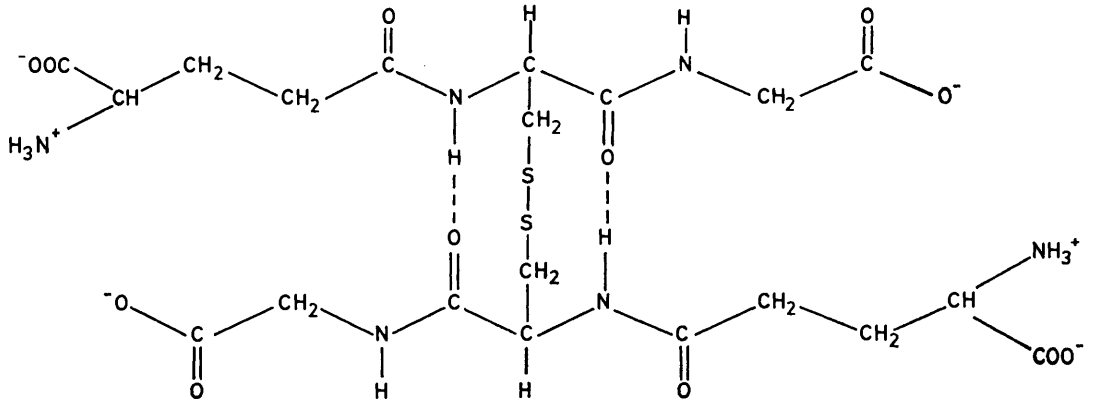
The Glu α-H–β-H coupling constants are both close to 6.2 Hz in the monomer, but 5.6 Hz in the dimer. This lower value suggests that neither β-proton is *trans* to the α-proton, which is in turn sterically consistent with the presence of some interaction between the Glu NH_3^+ and the Gly COO^- residues either as proposed above or due to steric considerations.

* $1° = (\pi/180)$ rad.

Finally, when the monomer is dissolved in H_2O and the solvent resonance is radio-frequency saturated, two amide proton resonances are observed with substantially reduced intensity due to saturation transfer by proton exchange. However, when the dimer is observed under similar conditions only the Cys-NH resonance behaves in this way. The Gly-NH resonance, which may be readily identified by its coupling patterns, is appreciably less affected, as would be expected if these protons were involved in hydrogen bonds.

Furthermore, previous studies of cyclohexapeptides [16,17] in water and dimethyl sulphoxide have shown these systems to possess a favoured conformation in which two antiparallel extended peptide segments are bridged by two transannular hydrogen bonds

All the above observations imply that structure (A) is present.



(A)

Proteins.—Earlier studies [5,6] have shown that a single-libration model can explain many observed protein T_1 values at frequencies between 14 and 68 MHz, and n.O.e values obtained at up to 45 MHz. However, two queries have since arisen. One is a suggestion by Allerhand [18] that the same results may be explained in terms of a rigid rotor with unexpectedly long α-C–H bonds. The second is that the single-libration theories predict a sharp increase in n.O.e. for resonances in small proteins at very high observation frequencies, and also for larger proteins at somewhat lower frequencies. Such measurements are now possible,[19] and we have therefore attempted them in order to test the single-libration theory more rigorously, and to compare it with the present theory.

In all three theories there is no problem in explaining how T_1 and T_2 vary with observation frequency. The crucial test lies with the values of n.O.e. Allerhand's rigid-rotor theory predicts that when the observation frequency is much greater than $\tau_R/2\pi$, the n.O.e. should be 1.15, independent of the CH bond length. Table 3 compares our experimental results for identifiable α-carbon resonance groups in various proteins with our theoretical results. It is evident that the single-libration theory predicts values of n.O.e. considerably larger than those actually observed, but also that the observed values of n.O.e. are significantly larger than those predicted for a rigid rotor. The two-libration theory gives better results than for a single-libration, because it does not artificially exclude the possibility of some internal or anisotropic rotational motions of intermediate rate, with the right correlation time to return the n.O.e. to a lower value. However, it works only by using rather large values of τ_R, from which we may conclude that in real proteins there is a still greater variety of types of slow motion than is allowed for in the present theory.

Conclusions.—The double-libration theory was devised in sufficiently general terms to be applicable to most molecules, and the above evidence shows that it withstands rigorous experimental testing better than other theories published to date. It yields sufficient detail to begin to allow some time-resolved conformation analysis of highly non-rigid molecules, although it requires further development and better experimental data before it can be used with confidence on proteins.

TABLE 3

Protein α-carbon: nT_1 and n.O.e. values, motional frequencies, and amplitudes from ^{13}C relaxation data

	Resonance frequency												Motion amplitude/frequencies [f]				
	15.2 MHz				22.6 MHz				45.2 MHz								
	nT_1 (obs.)	nT_1 (calc.)	n.O.e. (obs.)	n.O.e. (calc.) [e]	nT_1 (obs.)	nT_1 (calc.)	n.O.e. (obs.)	n.O.e. (calc.) [e]	nT_1 (obs.)	nT_1 (calc.)	n.O.e. (obs.)	n.O.e. (calc.) [e]	τ_G(ps)	X(rad)	τ_S(ps)	θ(rad)	τ_R(ns)
Ribonuclease-A	35.0 [a]	35.0		1.42					150.0 [b]	150.0	1.40	1.26	60	0.35	7 000	0.55	20
Myoglobin [c]	36.0	39.0	1.2	1.4 (1.3)	73.0	73.0	1.3	1.3 (1.3)					60	0.35	7 000	0.65	30
Bovine serum albumin [d]	100.0	82.0		1.21					570.0	573.0	1.30	1.32 (1.5)	60	0.35	20 000	0.47	130

[a] Results from ref. 20. [b] Results from ref. 5. [c] Results from ref. 21. [d] Results from ref. 22. [e] Figures in parentheses refer to n.O.e. values calculated using the single-libration theory.[5] [f] See footnote *f* of Table 1.

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