atoms was involved in hydrogen bonding. A similar scheme for a guanine-O^4-methyluracil mispair has also been proposed.[4] In order to form this pair the torsion angle ϕ [N(3)-C(4)-O(4)-C(7)] must be rotated at least ±80° (see Figure 4a) from the syn-periplanar conformation to alleviate steric clash between the C(7) methyl group and the guanine O(6) atom, particularly. Crystal structure determinations of and ab initio quantum mechanical calculations on many compounds containing monomethoxyphenyl moieties have shown that the planar conformations ($\phi = \pm 180°$) are easily accommodated by allowing the two methyl protons to straddle the ortho ring protons, thus avoiding unfavorable proton-proton interactions.[29] It is likely that the methoxy group will assume the anti-periplanar conformation in the guanine-O^4-methyluracil pair as an in-plane methoxy group allows maximum conjugation between the aromatic π electrons and the lone-pair electrons of O(4). However, the methoxy group of a guanine-O^4-methylthymine base pair must take an electronically less favorable (out of plane) conformation to avoid a collision between the C(5) and C(7) methyl groups that arises when ϕ approaches 180°. A second base-pairing model (Figure 4b) requiring a "wobble" of the pyrimidine base and the involvement of the O(4) atom is not as likely since the methoxy O(4) oxygen atom is expected to be less favored than the keto O(2) oxygen atom in hydrogen bonding.

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Summary

The conformations of the two independent molecules of O^4-methyluridine are similar. They display the C(3') endo sugar pucker, anti disposition of the base, and gauche$^+$ conformation about the C(4')-C(5') bond. However, the geometries of the pyrimidine bases show some striking differences. The pyrimidine ring of molecule A is in a twist-boat conformation, while that of molecule B is flat. Bonding differences are seen in the C(7)-O(4)-C(4)-C(5)-C(6) half of the base. These differences are attributed to the monopole-induced dipole interactions between the ribose ring O(4') oxygen atom and a neighboring pyrimidine base of A.

Acknowledgment. We gratefully thank NSERC, Canada (A6434), the National Institutes of Health for a research grant (GM-17378), and the College of Agricultural and Life Sciences of the University of Wisconsin—Madison for their continued support.

Registry No. O^4-Methyluridine, 34218-77-4.

Supplementary Material Available: Tables of anisotropic thermal parameters and observed and calculated structure factors (9 pages). Ordering information is given on any current masthead page.

An Examination of Relaxation Reagents for Conformational Analysis of Peptides in Aqueous Solution

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Abstract: Second-order rate constants have been measured for proton spin-lattice relaxation of protons of peptides and model amides in water, catalyzed by 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) and the *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEEDTA) complexes of Gd^{3+} and in some cases Cr^{3+}. Examined were 2-azacyclononanone, 2-azacyclohexanone, Ac-Pro-NHMe, Ac-Sar-NHMe, cyclo(His-Asp), and cyclo(Gly-Pro-D-Gln)$_2$. The effectiveness of the nitroxyl as a relaxation reagent in water reflects primarily the degree of exposure of the observed proton to the external environment, as previously assumed, although there is some evidence of CONH···O-N< association in dimethyl sulfoxide. In contrast, the Gd^{3+} complex shows affinity for carbonyl oxygen and can be used to distinguish cis from trans isomers at peptide bonds to proline and N-alkyl amino acid residues. The specificity of the Gd complex is enhanced in methanol and lost in dimethyl sulfoxide. CrHEEDTA was observed to show some selective effects not readily explained. HEEDTA complexes of Eu^{3+}, Ho^{3+}, and Dy^{3+} were also prepared.

The effectiveness of paramagnetic species in inducing nuclear magnetic relaxation depends on distance, so that paramagnetic cosolutes can be used to determine, by relaxation effects, the periphery of a folded chain molecule. For this kind of study of peptides we have preferred nitroxyls[1-3] as the paramagnetic species, and we have hesitated to use transition metal or lanthanide ions because chelation with less stable conformations of the peptides could distort the interpretation of differential relaxation effects. However, chelation by peptide might be less likely when the solvent is water and the ion is already associated with a good complexing agent. Therefore, we compared the specificities of a nitroxyl, 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo), and a neutral lanthanide complex, gadolinium *N*-(2-hydroxyethyl)ethylenediaminetriacetate (HEEDTA), in inducing spin-lattice relaxation of protons of several peptide models in aqueous solutions. The quantities measured were the second-order rate constants for relaxation catalyzed by the reagent. For *N*-acetylsarcosine *N*-methylamide we explored the dependence of the relaxation rate constants on peptide concentration in water, and we used the gadolinium complex in methanol and dimethyl sulfoxide as well. We also report some results with the HEEDTA complex of chromium(III).

Experimental Section

Materials. *N*-(2-Hydroxyethyl)ethylenediaminetriacetic acid (HEEDTA), 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo), 2-azacyclononanone, 2-azacyclohexanone (2-piperidone), and dysprosium, europium, gadolinium, and holmium oxides were obtained from Aldrich. Cyclo(His-Asp)[4] and cyclo(Gly-Pro-D-Gln)$_2$[5] have been reported previously.

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Table I. Rate Constants, L mol^{-1} s^{-1}, for Spin-Lattice Relaxation of Protons in Cyclo(Gly-Pro-D-Gln)$_2$ by Paramagnetic Reagents in Water[a]

proton	Tempo	GdHEEDTA	CrHEEDTA
Pro α	470	11000	4600
Pro δ	700	7700	2000
Gln α	500	12000	1600
Gln γ	500	8800	2200
Gly α (1)	880	11000	2500
Gly α (2)	980	11000	1900
Gln NH (backbone)	480	9200	2500
Gly NH	330	8200	1900
Gln NH$_2$ (*E*)	540	7500	1900
Gln NH$_2$ (*Z*)	520	17000	2600

[a] Peptide concentration 40 mM; temperature 7 °C. Rate constants were obtained from four to six T_1 measurements at different reagent concentrations; correlation coefficients >0.98. Tempo concentrations up to 6 mM, GdHEEDTA to 0.5 mM, and CrHEEDTA to 1.5 mM were used. N-H proton relaxation was studied in H_2O and C-H in D_2O.

N-Acetylproline *N*-methylamide and *N*-acetylsarcosine *N*-methylamide were prepared in this laboratory by using standard methods, by Anita Go.

HEEDTA–lanthanide complexes were prepared by boiling a slight excess of the oxide with HEEDTA in water until all but the excess oxide dissolved, filtering the solution, evaporating the filtrate, and recrystallizing the residue from water or water–2-propanol. The Cr(III) complex was prepared by Cr(II)-catalyzed reaction of HEEDTA and Cr(III) chloride and isolated by ion exchange on Dowex 50.[6] The results of combustion analyses of the HEEDTA complexes are given below.

		% C	% H	% residue (M_2O_3)
CrHEEDTA·H_2O	calcd	33.90	4.80	21.47
	found	33.32	5.42	21.21
DyHEEDTA·H_2O	calcd	25.80	3.65	40.22
	found	24.90	3.63	40.24
EuHEEDTA·$2H_2O$	calcd	25.90	4.00	38.01
	found	25.61	4.00	38.14
GdHEEDTA·$3H_2O$	calcd	24.70	4.32	37.20
	found	24.53	4.09	36.26
HoEDTA·H_2O	calcd	25.70	3.64	40.47
	found	25.57	3.74	39.74

Methods. NMR data were collected by using a Nicolet NT-300 spectrometer operating at 300 MHz, using a 4000-Hz sweep width and a 16K data table. Relaxation rates were measured by the inversion–recovery method, using a complex 180° pulse and alternating phases, except for studies of exchangeable amide N–H protons, where H_2O was required as the solvent. For H_2O solutions T_1 was measured by using the saturation-recovery method, with detection by the Redfield 2–1–4–1–2 pulse sequence, as described previously.[3] T_1 values were calculated by using the Nicolet software. $1/T_1$ was determined in each case in the absence of relaxation reagent and at four or more concentrations of the reagent. The corresponding second-order rate constants were calculated by using linear least-squares analysis.

Aqueous solutions of cyclo(Gly-Pro-D-Gln)$_2$ were studied at 7 °C to minimize interference between HDO and amino acid α-proton resonances in D_2O. Other data were taken at ambient temperature. H_2O solutions contained 20% D_2O for the field-frequency lock; 1% acetic acid was present to hold the pH near the proton exchange rate minimum.

Results

Initial experiments comparing 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) with paramagnetic HEEDTA complexes were made with the cyclic hexapeptide, cyclo(Gly-Pro-D-Phe)$_2$. This was chosen for its water solubility, good separation between backbone proton resonances, and the fact that it exists in water predominantly in the form with all peptide bonds trans. Second-order rate constants for N–H and α-proton relaxation by Tempo, GdHEEDTA, and CrHEEDTA are given in Table I. The data show that GdHEEDTA is 10–20 times more effective in inducing spin–lattice relaxation than is the nitroxyl and that Tempo and GdHEEDTA have different specificities. The nitroxyl distinguishes the Gly α-protons from those of Gln and Pro; in contrast, GdHEEDTA effects the four α-protons equally. The nitroxyl does not distinguish the *E* and *Z* N protons of the glutamine primary amide side chain, but GdEEDTA relaxes the proton cis to the carbonyl oxygen more than twice as much as the proton trans to the carbonyl oxygen.

Table II. Rate Constants (L mol^{-1} s^{-1}) for Spin-Lattice Relaxation of 2-Azacyclononanone N-H Proton by Paramagnetic Reagents in H_2O, 23 °C[a]

	Tempo	GdHEEDTA	CrHEEDTA
cis[b]	240	7300[c]	1400
trans	260	1800	900

[a] Measurements were made at four concentrations of Tempo to 5.5 mM and CrHEEDTA to 1 mM and six concentrations of GdHEEDTA to 0.3 mM. Lactam concentration was 100 mM. [b] Proton cis to carbonyl oxygen; cis form of lactam. [c] The rate constant for 2-piperidone, cis form only, was measured to be 10 000 L mol^{-1} s^{-1}.

Table I also shows that CrHEEDTA is more effective than Tempo but less effective than GdHEEDTA. There is a significant if small difference in its effects on the *E* and *Z* N protons of the primary amide group. An interpretation of the high rate constant for the Pro α-proton is not obvious.

The different selectivities of Tempo, GdHEEDTA, and CrHEEDTA for *E* and *Z* N protons of primary amides, revealed in the cyclic hexapeptide experiments, were tested in the simpler case of 2-azacyclononanone. This lactam exists in solution in both cis and trans forms.[7] The results are given in Table II. Again, the nitroxyl makes no distinction between the N protons cis (*Z*) and trans (*E*) to the carbonyl oxygen, but the Gd complex affects the *Z* proton 4 times more strongly. The Cr complex is slightly (1.5×) more effective toward the *Z* protons. Assignment of the large Gd effect to the *Z* proton is confirmed by the comparable rate constant measured for 2-piperidone (Table II, footnote *c*), in which the amide bond is constrained to be cis so that the N–H proton is cis to the carbonyl oxygen (*Z*). The lactam data very strongly indicate coordination of GdHEEDTA to carbonyl oxygen in water.

These results with GdHEEDTA prompted a study of *N*-acetyl-*N'*-methylsarcosine amide. Although cis–trans isomerism (with reference to the peptide backbone) at the X–Pro peptide bond can be determined by a ^{13}C chemical shift correlation,[8,9] there has been no clear way to make the same determination for peptides of acyclic N-alkyl amino acids. Table III shows the relaxation rate data from a series of measurements on Ac-Sar-NHMe.

The first two columns of Table III show that the activity of the nitroxyl in water toward a given class of proton is the same in either isomer. GdHEEDTA is again 10–30 times as effective in inducing relaxation as is the nitroxyl, and it makes a clear distinction between cis and trans isomers. The protons of the groups attached cis to the carbonyl oxygen, i.e., the α-methylene protons in the trans form and the *N*-methyl protons in the cis form, are much more sensitive to the gadolinium reagent than those in the groups trans to the carbonyl oxygen. For the methylene protons the difference is a factor of 2.3, but for the *N*-methyl protons the factor is smaller, about 1.6. GdHEEDTA-induced relaxation of the terminal *N'*-methyl group is also isomer dependent, even though it is cis to its carbonyl oxygen in both isomers. The *N'*-methyl in the trans form has the higher rate constant. These effects were found to be independent of the peptide concentration in water, at least in the range 0.02–0.2 M.

In methanol, the rate constants for relaxation by GdHEEDTA are larger, and the distinctions between the two isomers of Ac-

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Table III. Rate Constants for Spin-Lattice Relaxation of *N*-Acetylsarcosine *N*-Methylamide Protons by Paramagnetic Reagents[a]

	reagent:	Tempo/D_2O[b]		Tempo/Me_2SO-d_6[b]		GdHEEDTA/D_2O[c]			GdHEEDTA/ CD_3OD, 100 mM	GdHEEDTA/ Me_2SO-d_6, 100 mM
proton class[d]	concn of peptide: *n*:[e]	20 mM 4	100 mM 5	20 mM 4	100 mM 5	20 mM 5	100 mM 6	200 mM 6	5	4
CH_2 cis		270	280	250	210	3400	3700	3200	11000	5900
CH_2 trans		260	280	230	210	7700	8800	7300	47000	5400
$>NCH_3$ cis		230	230	220	180	4000	4300	3900	9200	2900
$>NCH_3$ trans		230	230	240	210	2500	2700	2300	4700	2600
$HNCH_3$ cis		210	220	220	190	2400	2800	2400	4000	3300
$HNCH_3$ trans		210	210	210	190	3200	3500	3100	10000	3500
CH_3CO cis		220	230	220	190	4300	4800	4200	12000	3100
CH_3CO trans		200	190	220	190	4600	5100	4300	18000	2800
NH cis				360	300					9800
NH trans				350	330					7800

[a] L mol^{-1} s^{-1} at 23 °C. Obtained from least-squares plots of $1/T_1$ vs. reagent concentration; n = number of concentrations used; correlation coefficients >0.99. [b] Concentration range 0–6 mM. [c] Concentration range 0–0.5 mM in D_2O and 0–0.2 mM in organic solvents. [d] Cis and trans refer to the peptide carbon backbone across the Ac-Sar bond. [e] Number of concentrations at which T_1 was measured.

Table IV. Chemical Shifts of *N*-Acetylsarcosine *N*-Methylamide Protons[a]

proton class	D_2O	Me_2SO-d_6	CD_3OD
CH_2 cis	4.16	3.88	4.04
CH_2 trans	4.04	3.82	4.00
$>NCH_3$ cis	2.92	2.74	2.91
$>NCH_3$ trans	3.12	2.95	3.08
$HNCH_3$ cis	2.78	2.60	2.77
$HNCH_3$ trans	2.75	2.56	2.74
CH_3CO cis	2.08	1.90	2.05
CH_3CO trans	2.18	2.00	2.14

[a] Reference is sodium trimethylsilylpropionate in water and Me_4Si in organic solvents.

Table V. Chemical Shifts and Rate Constants (L mol^{-1} s^{-1}) for Spin-Lattice Relaxation of *N*-Acetylproline *N*-Methylamide Protons by GdHEETA in D_2O, 23 °C[a]

	H^{α}		$HN-CH_3$		CH_3CO	
	cis	trans	cis	trans	cis	trans
chemical shift	4.64	4.49	2.93	2.88	2.18	2.27
rate constant	1300	3000	1100	1600	1700	1900

[a] Eight measurements in GdHEEDTA concentration range 0–0.8 mM; correlation coefficients 0.99. AcProNHMe concentration = 60 mM. Chemical shift reference sodium 3-(trimethylsilyl)-propionate.

Table VI. Rate Constants for Spin-Lattice Relaxation of Protons in Cyclo(His-Asp) Catalyzed by Paramagnetic Reagents in D_2O[a]

	Tempo		GdHEEDTA	
proton	pH 2	pH 9	pH 2	pH 9
imidazole 2	430	520	5200	8900
imidazole 4	410*	490	5800	7200
His α	580	500	6300	11000
Asp α	610*	370	23000	51000
His β	530	350	9000	17000
Asp β	510	400	24000	44000

[a] L mol^{-1} s^{-1}, 7 °C; peptide concentration = 40 mM. Rate constants obtained from five measurements; correlation coefficient >0.98 except >0.95 in two cases indicated by an asterisk. Tempo concentrations to 5 mM and GdHEEDTA to 0.2 mM.

Sar-NHMe are also larger. The methylene protons are distinguished by a factor of 4, the *N*-methyls by a factor of 2, and the *N'*-methyls by a factor of 2.5. The acetyl methyls are also distinguished; as with the *N'*-methyls, the acetyl in the trans isomer is more sensitive.

In dimethyl sulfoxide solution the GdHEEDTA-induced relaxation is not isomer dependent, although there are significant differences in the rate constants for the different classes of protons in Ac-Sar-NHMe. The rate constants for the nitroxyl-induced relaxation in Me_2SO are about the same as those for aqueous solutions. With both nitroxyl and GdHEEDTA in Me_2SO the N protons show dignificantly higher rate constants than do carbon-bound protons.

The chemical shift data in Table IV may be of interest, since the relaxation data are unequivocal in assigning cis and trans isomers. In all three solvents, the *N*-methyl protons of the cis isomer are 0.2 ppm upfield of those of the trans form. The methylene protons of the trans isomer are 0.04–0.12 ppm upfield of those in the cis form. Protons of the group attached to amide nitrogen and cis to the carbonyl oxygen thus find themselves in a region of average shielding by the carbonyl. However, the effects are not large, and it may be risky to use the chemical shifts to make assignments.

Table V shows that GdHEEDTA can be used also to distinguish between cis and trans backbones in Ac-Pro-NHMe. As with the sarcosine derivative, GdHEEDTA relaxes the α-protons in the trans form, which are closer to the carbonyl, twice as effectively as those of the cis form.

To obtain some idea of the effect of charged groups on relaxation by nitroxyl and GdHEEDTA, we measured the relaxation

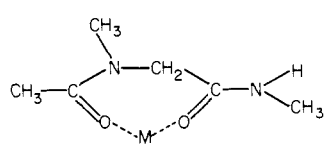
induced by these reagents in anionic and cationic forms of cyclo(L-His-L-Asp). The data are given in Table VI. Contrary to a previous suggestion,[4] protonation of imidazole does not appear to have a large effect on the rate of its relaxation by nitroxyl, nor does deprotonation of the aspartic acid side chain have much affect the nitroxyl rate constants of its β-protons. However, the aspartic acid residue has a definite affinity for the Gd complex, which is obviously stronger when the side chain carboxyl is ionized. Similarity of the rate constants for GdHEEDTA relaxation of the Asp α- and β-protons suggests chelation and stronger chelation in the anion. The effects of this tighter binding in the anion are seen on the relaxation of the nearby His α- and β-protons as well.

Discussion

Gadolinium HEEDTA. The effects of the lanthanide shift and relaxation reagents on NMR spectra occur through coordination of the lanthanide ion with the subject molecules, usually at oxygen. In acidic aqueous solutions shift and relaxation data show that hydrated lanthanide ions coordinate with oxygen anions, e.g., carboxylate and phosphate anions. In alkaline solution, lanthanide ions have been used in the form of the monoanionic ethylenediaminetetraacetic acid complexes ($LnEDTA^-$), and the effects of these are also explained by coordination with carboxylate and phosphate groups. Gadolinium ions exert relaxation effects that are proportional to the average inverse sixth power of the distance between the subject nucleus and the Gd center $\langle r^{-6} \rangle$, and this effect has been proposed for determining peptide sequence by using $GdEEDTA^-$, since the relaxation induced by the reagent decreases with distance from the carboxylate terminus.[10]

In this work we observe stereospecific relaxation effects induced by the aqueous gadolinium (hydroxyethyl)ethylenediaminetriacetic acid complex in molecules that lack carboxylate or phosphate anionic groups. These effects indicate that GdHEEDTA (presumably also Gd^{3+} or $GdEDTA^-$) can exert significant and useful effects by coordination at peptide carbonyl oxygen. That GdHEEDTA is much more effective in relaxing the *Z* amide proton of the cis form of azacyclononanone than in relaxing the *E* proton of the trans isomer is most satisfactorily interpreted to indicate that the GdHEEDTA associates with the amide carbonyl oxygen and is thus closer on the average to the *Z* proton.

The differentiating effects of GdHEEDTA on the two forms of acetylsarcosine *N*-methylamide are also explained as a result of coordination to peptide carbonyls. The group that is attached to the amide nitrogen on the carbonyl oxygen side of the C–N bond is closer to the gadolinium ion than the group attached on the opposite side. It is to be noted that the catalytic rate constant difference between the cis and trans peptide forms is greater at the α-methylene protons than at the *N*-methyl protons. This may be the result of conformational preferences in the trans form of Ac-Sar-NHMe. If $\langle \phi \rangle$ is near 180°, the averaged distance of the methylene protons from the carbonyl oxygen in the trans form is less than the average for the protons of a freely rotating methyl group in the cis form. Alternatively, and more likely, the difference may be the result of chelation, and thus stronger binding, of gadolinium by the trans form. Most probably the greater sensitivity of the *N'*-methyl group ($-NH-CH_3$) in the trans isomer is the consequence of chelation by the trans form, since the relationship between this group and the nearest carbonyl to it is independent of the form of the Ac–Sar peptide bond. Chelation, shown below, would occur in conformations of the trans form with



ϕ near 60° or −60° rather than near 180°. This chelation is not possible in the cis form. Not only does chelation in general distort the conformational distributions but also different conformations will have different affinities for the lanthanide. These effects are certain to produce serious ambiguities in conformational interpretation of gadolinium-induced relaxation data with more complex flexible molecules.

Chelation of the lanthanide between side chain carboxyl and peptide carbonyl is probably the explanation of the very high rate constants for GdHEEDTA-induced relaxation of the aspartic acid α- and β-protons in cyclo(L-His-L-Asp) (Table VI).

In the experiments with Ac-Sar-NHMe, its concentration was 40–400 times the maximum concentration of GdHEEDTA used. The concentration independence of the second-order rate constants in water (Table III) indicates that although coordination may be important in producing the differences noted in relaxation rate constants, the fraction of Gd bound to peptide is not large. In methanol, which may be presumed to be less effective than water in solvating peptide or lanthanide, their association is probably greater, giving rise to the larger rate constants for relaxation and the larger differences between isomers for the sarcosine methyl and methylene groups. Since the differentiation at the *N'*-methyl is greater in methanol, and differentiation between the acetyl methyls also occurs, it seems likely that the contribution from chelate formation is also larger in methanol than in water.

In dimethyl sulfoxide GdHEEDTA does not distinguish between the cis and trans forms of Ac-Sar-NHMe. If the stereospecificity seen in water or methanol arises from coordination between gadolinium and peptide, then coordination between gadolinium and peptide carbonyl is probably not important in Me_2SO, probably because the solvent competes successfully for sites in the Gd coordination sphere. Since the rate constants for Me_2SO solution are of the same order of magnitude as for aqueous solutions, it seems that a large part of the Gd-induced relaxation in all three solvents could arise from random collisions between GdHEEDTA and peptide. However, GdHEEDTA in Me_2SO does apparently affect proton groups to an extent depending on their covalent structure, $N-H > CH_2 > CH_3$. One possible explanation of the higher rate constants for the N protons may be association by hydrogen bonding to the basic carboxylate ions of the complex. To explain the result alternatively, there could be a less sterically specific association of Gd with amide groups, perhaps through the π electrons, since the N–H is directly bonded to an amide function, the CH_2 protons are two bonds from two amide groups, and the CH_3 protons of each methyl are two bonds from one amide group. The hydrogen-bonding explanation seems more likely, since it can also apply to observations with the nitroxyl in Me_2SO (see below).

In distinguishing the cis and trans isomers of *N*-acetylproline *N*-methylamide in water, we found that GdHEEDTA also shows specificity resulting from association with one or both carbonyl oxygens, analogous to the observations with Ac-Sar-NHMe. What is puzzling, however, is that the rate constants for the acetyl methyls and *N'*-methyls of Ac-Pro-NHMe are half or less of those for the corresponding methyls of Ac-Sar-NHMe. Self-association of Ac-Pro-NHMe that restricts access of GdHEEDTA is a possible, but not strongly suggested, explanation.

Chromium(III) HEEDTA. The chromium(III) HEEDTA complex is expected to be relatively inert to substitution and the chromium ion to remain six-coordinate.[11] Chemical interaction with peptides should thus be much less than for GdHEEDTA. Nonetheless, CrHEEDTA does relax the amide proton in the cis form of azacyclononanone more efficiently than in the trans form, although the difference is much smaller than with GdHEEDTA. An explanation based on accessibility rather than coordination to carbonyl may be possible, but it is also possible that hydrogen bonding of N–H to CrHEEDTA may be involved.

2,2,6,6-Tetramethylpiperidinyl-1-oxy (Tempo). The effects of the nitroxyl on the amide proton of azacyclononanone in water and the *N*-methyl and α-methylene protons of Ac-Sar-NHMe in water or Me_2SO are not dependent on whether the configuration at the corresponding amide bonds is cis or trans. However, as

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with GdHEEDTA, the N protons are more sensitive to the relaxing agent in Me_2SO than are the carbon-bound protons. This is not fully consistent with the assumption we have been making in previous use of this relaxation reagent, that in basic (hydrogen bond accepting) solvents there is no specific association of the nitroxyl with peptide bonds. While there may be no specific association with –CONH– groups in water (see, for example, the cyclic hexapeptide data in Table I), or methanol, in the aprotic Me_2SO the hydrogen-bonding basicity of the nitroxyl may increase enough to make it compete detectably with solvent in forming hydrogen bonds to peptide N–H.

Experiments using the nitroxyl as a probe of cyclo(L-His-L-Asp) were carried out because previous results with angiotensin II and LHRH had suggested that the accessibility of the imidazole ring to nitroxyl might significantly depend on its state of ionization.[4] The present results do show a decrease in relaxation rate constant at C^2–H and C^4–H in the cationic form, but the effect is barely significant. The explanations in the case of the larger peptides will have to involve chain folding or intermolecular association.

Cyclo(Gly-Pro-D-Gln)$_2$. The backbone fonformation of cyclo(Gly-Pro-D-Gln)$_2$ most probably is constructed from two type II β-turns[5] and is similar to the backbones found in crystals of cyclo(Gly-Pro-D-Ala)$_2$[12] and cyclo(Gly-Pro-D-Phe)$_2$.[13] In this model, the N–H bonds of the Gly residues are directed approximately into the peptide ring, and the Gly C–H bonds and Pro C^δ–H bonds are directed out of the ring on its periphery. The C^α–H bonds of Pro and Gln and the backbone N–H bond of Gln are directed roughly perpendicular to the plane of the peptide ring. The nitroxyl data in Table VI show rate constants of 500 ± 50 L mol^{-1} s^{-1} for most of the protons exposed to the exterior, Pro α, Gln N, α, γ, and side chain NH_2 (There is no distinction by the nitroxyl between *E* and *Z* protons.) The sequestered Gly N–H proton is significantly less affected by the radical. The protons directed out of the ring at its periphery, Gly α and Pro δ, are relaxed with considerably greater efficiency. Although a quantitative explanation of the differential effects, particularly the enhanced sensitivity of protons at the periphery of the peptide ring, is not obvious on looking at the molecular models, it seems likely that the differences will eventually be shown to reflect the appropriately weighted distance averages $\langle r^{-6} \rangle$ for random collisions between the N–O radical center and the protons concerned.

GdHEEDTA does not distinguish among the α-protons of cyclo(Gly-Pro-D-Gln)$_2$, all of which are near two possible carbonyl coordination sites, and distinctions that might result from the different values of ϕ and ψ at each residue are probably blurred by overlapping effects of multiple sites of coordination. However, the Gly–Pro peptide bond in this peptide is trans, and there is a distinction between Pro α- and Pro δ-protons in the same sense as is observed for the trans form of Ac-Sar-NHMe, α more sensitive than N–CH_3 (=δ –CH_2). Unhappily, the ratio of the α and δ rate constants is much smaller in this more complex molecule, again probably because of multiple coordination sites. Although it is likely that GdHEEDTA can be used for assignment of stereochemistry at peptide bonds to N-alkyl amino acid residues in larger peptides, care in interpreting the relaxation data will be required if only one form is present.

GdHEEDTA and CrHEEDTA both differentiate the *E* and *Z* protons of the Gln side chain amide. There is a large difference in rate constants with Gd and a much smaller one with CrHEEDTA, similar to the observations with these reagents and azacyclononanone.

The striking feature of the CrHEEDTA–cyclohexapeptide data is the extraordinarily high rate constant observed for the Pro α-proton. More examples of specific relaxation effects by CrHEEDTA will be required before this observation can be explained. The only significant distinction of this proton in the model of cyclo(Gly-Pro-D-Gln)$_2$ is its cisoid relationship to the Gln N–H (ψ_{Pro} near 120°).

Summary. The experiments described indicate that Gd in an EDTA-like complex associates with peptide bond carbonyls in water or methanol and suggest that GdHEEDTA can be used in these solvents, but not in dimethyl sulfoxide, to distinguish cis–trans isomerism at peptide bonds to N-alkyl amino residues. The nitroxyl spin-label, Tempo, does not produce differential relaxation effects arising from specific association in water with the peptides and amides examined.

Acknowledgment. This work was supported by grants from the National Institute of General Medical Sciences, GM 26071 and GM 26071-02S.

Registry No. CrHEDTA, 15819-54-2; DyHEDTA, 77461-03-1; EuHEDTA, 77461-02-0; GdHEDTA, 74546-40-0; HoHEDTA, 74546-41-1; TEMPO, 2564-83-2; Ac-Pro-NHMe, 24847-46-9; Ac-Sar-NHMe, 24131-61-1; cyclo(L-His-L-Asp), 35507-96-1; cyclo(Gly-Pro-D-Gln)$_2$, 85724-50-1; 2-azacyclononanone, 935-30-8; 2-azacyclohexanone, 675-20-7.

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