^{17}O NMR Chemical Shifts as a Tool to Study Specific Hydration Sites of Amides and Peptides: Correlation with the IR Amide I Stretching Vibration

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A model of specific hydration sites of amides and peptides based on ^{17}O NMR chemical shifts is presented. Solvation of the amide hydrogen (NH) is shown to induce a very small modification of the shielding of the amide oxygen and thus can be neglected. On the contrary, long-range dipole–dipole interactions and specific hydration at the amide oxygen are demonstrated to induce large and specific modifications of the ^{17}O shielding constants. The significance of wave function overlap in hydrogen bonding interactions is emphasized. Experimental evidence is provided for cooperativity in hydrogen bonding of the bound molecules of water at the amide oxygen due to increased dielectric constant of the medium and further solvation with molecules of water. It is demonstrated that ^{17}O shielding constants can contribute valuable information on specific hydration of the peptide oxygen. Very good linear correlation between $\delta(^{17}O)$ and $\nu(CO)$ (the amide I stretching vibrational frequency) was found for different solvents which have varying dielectric constants and solvation abilities. For solvents in which two IR bands were observed the weighted mean of these bands was used. The relative advantages and disadvantages of both methods in studying and quantitating specific hydration at the amide and peptide oxygen are critically evaluated.

Introduction

Peptide and protein hydration is a dominant factor in the folding process and spatial stability of molecular structures of peptides and proteins and plays an essential role in protein–ligand interactions and in the mechanisms of peptide- and protein-mediated reactions.[1-3] However, contrary to numerous experimental reports on the solvation state of peptides and proteins in crystals,[4-9] knowledge of the specific hydration state of the peptide oxygens at a molecular level in aqueous solution is rather limited. NMR studies of peptide and protein hydration rely primarily on phenomena related to spin relaxation of the bulk water signal.[10,11] These experiments could not provide information on the location of the hydration molecules, although they provide evidence that at least part of the water associated with proteins is highly mobile, with residence times in the hydration sites in the subnanosecond range. Only recently a limited number of "long"-lived individual water molecules bound to specific hydration sites in small proteins in solution has been observed with an elegant use of 2D and 3D rotating frame Overhauser spectroscopy (ROESY) and heteronuclear 3D rotating frame Overhauser ^{15}N–^{1}H multiple quantum coherence (^{15}N–^{1}H ROESY–HMQC) spectroscopy.[12-17] Similar experiments with the peptide hormone oxytocin indicated that all parts of the molecule are exposed to the solvent.[15] However, no stable bound molecules of water could be identified for this peptide. Thus, a detailed solvation picture does not seem to be warranted.

NMR chemical shift has long been recognized as being extremely sensitive to local electron distributions, bond hybridization states, proximity to polar groups, nearby aromatic rings, and local magnetic anisotropies, and several empirical "rules" which relate shielding and molecular structure have been established.[18] The same success has not been forthcoming in attempting to find such generalizations for solvation effects and, more specifically, to hydration phenomena. In many cases, intermolecular effects on the ^{1}H chemical shifts are of the same order of magnitude as intramolecular effects, and this greatly complicates the quantitative evaluation of specific solvation phenomena. ^{13}C and ^{15}N chemical shifts are quite sensitive to hydrogen bonding interactions but the available data do not permit any generalizations in regard to of specific hydration phenomena.[19] ^{17}O chemical shifts were recognized as early as 1963 to be very sensitive to hydrogen bonding interactions;[20] however, it is only in recent years that this technique has received attention as a structural probe in amides and peptides.[21-28] The reasons

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for the difficulty in performing ^{17}O NMR experiments (low sensitivity, large line widths, and rolling base lines) are documented elsewhere.[29,30]

Fiat and co-workers[21,25] in a series of elegant investigations suggested a model for separating the various contributions to the ^{17}O nuclear shielding constants due to hydrogen bonding–hydration phenomena at various sites in the amide molecule. In this paper, we present an improved solvation model of amides and peptides based on ^{17}O NMR chemical shifts. (Preliminary results of which have been reported[31]). It is demonstrated that both long-range dipole–dipole interactions and specific hydrogen bonds due to solvation of molecules by H_2O at the amide oxygen induce significant and specific modifications of the ^{17}O shielding constants which are larger than originally considered.[21,25] The significance of wave function overlap in hydrogen bonding interactions is emphasized. Further, experimental examples of cooperativity in hydrogen bonding of the bound molecules of water are presented. Solvation of the amide hydrogen is shown to induce a very small modification of the shielding of the amide oxygen and thus can be neglected. Very good correlation of $\delta(^{17}O)$ *vs* $\nu(CO)$ (the amide I stretching vibrational frequency) was found for different solvents which have varying dielectric constants and solvation abilities.

Experimental Section

^{17}O-NMR spectra were recorded on a Bruker AM-400 spectrometer operating at 54.48 MHz. The spectrometer was equipped with a high-resolution probe accepting 10-mm sample tubes. The field was optimized using D_2O. No field lock was used during data acquisition. The chemical shifts (ppm) were determined relative to external 1,4-dioxane (+0.2 ppm relative to H_2O at 303 K) determined in a separate replacement experiment. Their errors were estimated $<\pm 0.5$ ppm for concentrations above 0.1 M and $<\pm 1$ ppm for concentrations between 0.1 M and 0.01 M. Chemical shifts were corrected for the bulk magnetic susceptibility effect

$$\delta_{corr} = \delta_{obs} + \frac{2\pi}{3}[\chi_{1,4\text{-dioxane}} - \chi_{solvent}] \quad (1)$$

for a cylindrical sample perpendicular to the magnetic field, where $\chi_{solvent}$ and $\chi_{1,4\text{-dioxane}}$ are the bulk magnetic susceptibilities of the solvent and of the reference compound 1,4-dioxane, respectively. Data acquisition parameters: spectral width 50 KHz, 90° pulse 32 μs, quadrature phase detection, $T_{acq} \sim 5T_2$, zero filling to 4K before Fourier transform. The number of scans was between 20 000 and 100 000 for concentrations above 0.1 M and between 100 000 and 3 000 000 for concentrations between 0.1 and 0.01 M. Acoustic ringing effects[32] were alleviated using a preaquisition delay time Δt = 30–100 μs. For the experiments in aqueous solutions ^{17}O-depleted water (YEDA, ^{17}O content = 10^{-3}%) was used to circumvent the problem of dynamic range.

IR spectra were recorded at 303 K on a Nicolet MX-S FTIR spectrometer with a 250-μm cell equipped with CaF_2 windows, using solutions of 10–100 mM concentration. Each spectrum was recorded with resolution 2 cm^{-1}, and it was the result of 16 scans accumulation. The solvent absorption bands were suppressed by subtracting the solvent spectrum from that of the solution spectrum, which was recorded in the same cell.

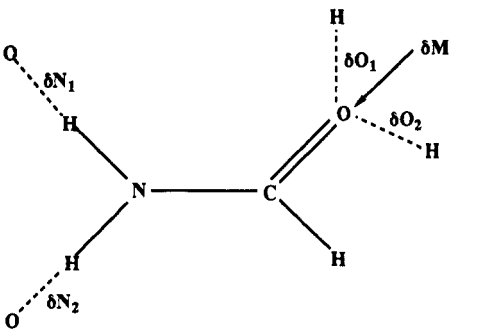

Figure 1. Hydration model of amides based on ^{17}O shielding. δO_1 and δO_2 are the contributions to the observed chemical shift due to the first and second oxygen lone pairs respectively participating in a hydrogen bond. δN_1 and δN_2 are the contributions due to solvation of the first and second amide nitrogen protons.[21]

Results and Discussion

1. ^{17}O NMR Chemical Shifts and Specific Solvation Phenomena in Amides. The model of amide solvation suggested by Fiat *et al.*[21,25] postulates the separation of the ^{17}O nuclear shielding into five contributions. The model is based on two hypotheses: (i) the effects of hydrogen bonding at the various sites act independently of one another, and (ii) alkyl substitution at the amide nitrogen does not affect the observed chemical shift δ_{obs}, *i.e.*, $\delta M(FOR) = \delta M(NMF) = \delta M(DMF) = 323$ ppm, where δM is the chemical shift of the amide oxygen in the absence of hydrogen bonding interactions. δ_{obs} therefore is given by

$$\delta_{obs} = \delta M + \delta O_1 + \delta O_2 + \delta N_1 + \delta N_2 \quad (2)$$

where δO_1 and δO_2 are the contributions to the observed chemical shift due to the first and second oxygen lone pairs, respectively, participating in a hydrogen bond and δN_1 and δN_2 are the contributions due to the first and second nitrogen proton participating in a hydrogen bond (Figure 1). From dilution studies in different solvents with high dielectric constants the following values were suggested for formamides:

$$\delta M = 323,\ \delta O_1 = -22,\ \delta O_2 = -31,\ \delta N_1 = 9,\ \text{and}\ \delta N_2 = 2\ \text{ppm} \quad (3)$$

For acetamides:

$$\delta M = 340,\ \delta O_1 = -22,\ \delta O_2 = -32,\ \delta N_1 = 10,\ \text{and}\ \delta N_2 = -9\ \text{ppm} \quad (4)$$

(a) Substituent Effects. It is well known that the ^{17}O chemical shifts of ketones, aldehydes, alkanoid acids, and alcohols depend upon substituent effects up to several

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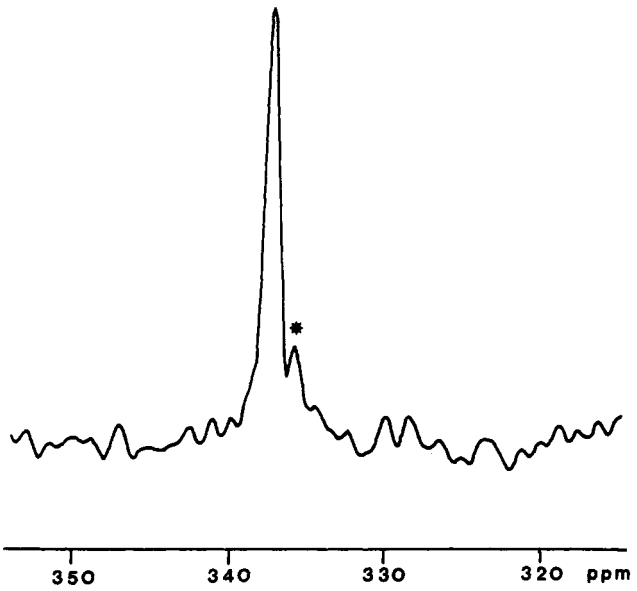

Figure 2. ^{17}O NMR spectrum (54.48 MHz) at natural abundance of NMF in toluene, 10-mm sample tubes, quadrature detection, from use of the normal 90° pulse (~25 μs) with a preaquisition delay time Δt ~ 100 μs in order to eliminate acoustic ringing problems, 303 K, concentration 30 mM, number of scans 2 860 000, total experimental time ~11.5 h. Prior to transformation the FID was multiplied with a line broadening function LB ~20 Hz. Chemical shifts are measured relative to 1,4-dioxane (+0.2 ppm relative to H_2O at 303 K) determined in a separate replacement experiment. The resonance marked by the asterisk is, presumably, due to the cis isomer; its intensity corresponds to a population of ca. 9% which is in excellent agreement with the integral of the cis isomer derived from ^1H NMR.

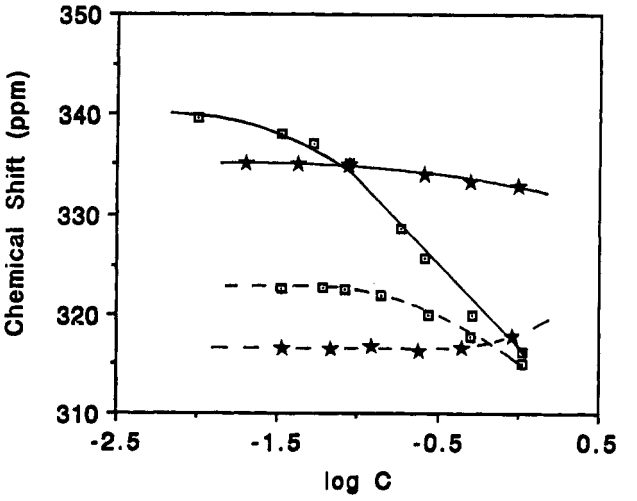

Figure 3. ^{17}O NMR chemical shifts of NMF (□) and DMF (*) as a function of logarithm of concentration, log C, in toluene (—) and CH_2Cl_2 (---).

bonds away.[29,30,33,34] Therefore, the hypothesis that alkyl substitution of the amide nitrogen does not affect the ^{17}O chemical shifts should be verified experimentally. This was achieved by recording the ^{17}O NMR spectra of DMF, NMF, DMA, and NMA over a range of concentration (1 M to 10^{-2} M) in several solvents (Figures 2 and 3). On decreasing the concentration in the apolar and low dielectric constant solvents *n*-hexane, CCl_4, and toluene, the NMR signal indicates strong deshielding (shift to high frequency) for NMF due to the rupture of intermolecular interactions between the amide NH group of one with the CO group of another molecule. N-Monosubstituted amides in solution can exist in different hydrogen bond states such as monomer, dimer, ..., *n*-mer. For linear *n*-merization the observed average shift due to fast chemical exchange (on the NMR time scale) would then be given by[35]

$$\delta = (M_1/C)\,\delta_1 + (M_2/C)\,\delta_2 + \ldots + (M_n/C)\,\delta_n \qquad (5)$$

where δ_1, δ_2, ..., and δ_n are the average shifts of the amide oxygens in the monomer, dimer, ..., and *n*-mer, respectively, and $M_1, M_2, \ldots, M_n$ are the concentrations of the monomer, dimer, ..., and *n*-mer, respectively. *C* is the total concentration of the solute given by

$$C = \sum_{i=1}^{i=n} i K_i^{i-1} M_1^{\,i} \qquad (6)$$

Further,

$$K_n = M_n/M_1^{\,n} \qquad (7)$$

where $K_1, K_2, \ldots$, and K_n are the association constants for dimerization, trimerization, ..., and *n*-merization, respectively. The linear *n*-merization model would leave a single nonbonded amide oxygen at the end of each polymer chain with chemical shift equal to that in the monomer state. Thus

$$\delta_n = \frac{\delta_1 + (n-1)\delta_{\text{H-bonded}}}{n} \qquad (8)$$

where $\delta_{\text{H-bonded}}$ is the chemical shift of the amide oxygen participating in a hydrogen bond interaction. The fitting of the experimental dilution data of Figure 3 was accomplished by taking advantage of two features of such plots. As log *C* decreases the chemical shift approaches a constant value. This limiting value is the chemical shift of the monomer δ_1. Moreover, a virtually linear portion appears at higher values of log *C*[35] (see the dependence of $\delta(^{17}O)$ of NMF in toluene, Figure 3). Having noted that the slope of the corresponding linear portion of the theoretical plots may be adjusted by varying δ_n, we may find the best value of this final parameter by adjusting it until the slope of the theoretical line corresponds with that of the experimental curve. This fitting process has been carried out for $n = 2$–4 and has led to the parameter value $n = 2$. The monomer chemical shifts appear to be practically independent of *n*, contrary to association constants which were found to be strongly dependent upon *n*. The chemical shifts of monomer NMF and NMA in a variety of solvents are shown in Table 1.

^{17}O NMR chemical shifts of DMF (Figure 3) and DMA indicate a significantly smaller concentration dependence. The difference in the chemical shift of NMF and DMF at infinite dilution in *n*-hexane, CCl_4, and toluene (Table 1) is rather small, +4 to +6 ppm, and practically not dependent on the solvent. Since both molecules are expected to be in the monomeric state at infinite dilution in the above solvents, it is evident that the difference in the chemical shift can be attributed to the substituent effect of the methyl group of the amide nitrogen. This effect is in the same direction but smaller in magnitude

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Table 1. Solvent Effects on the ^{17}O Chemical Shifts[a] and Amide I, ν(CO), Stretching Vibration of Amides

	NMF		DMF		NMA		DMA	
solvent	NMR (ppm)	IR (cm^{-1})	NMR (ppm)	IR (cm^{-1})	NMR (ppm)	IR (cm^{-1})	NMR (ppm)	IR (cm^{-1})
n-hexane			347.2 (347.1)	1695[b]		1697[b]		1672[b]
CCl_4	343.5 (343.7)	1701[c]	337.8 (337.8)	1685[c]			353.5 (353.7)	1658[c]
toluene	340.0 (340.0)	1699[c]	335.3 (335.3)	1686[c]				
CH_2Cl_2	323.3 (323.6)	1688[b]	316.5 (316.8)	1674[b]	326.5 (326.8)	1673[b]	332.3 (332.5)	1640[b]
$CHCl_3$	319.5 (319.8)	1687[c]	310.3 (310.5)	1672[c]	320.3 (320.5)		325.6 (325.9)	1633[c]
acetone	329.0 (328.7)	*d*	327.4 (327.1)	*d*	333.8 (333.5)	*d*	343.9 (343.6)	*d*
CH_3CN	323.1 (323.0)	1689[b]	320.2 (320.1)	1676[b]	327.4 (327.4)	1674[b]	336.2 (336.1)	1644[b]
DMSO	320.9	1679[b]	320.4	1671[b]	326.4	1667[b]	338.3	1638[b]
EtOH	296.0 (295.9)		299.3 (299.2)		297.0 (297.0)		311.4 (311.3)	
MeOH	290.8 (290.6)	1670[b]	292.5 (292.3)	1662[b]	290.5 (290.3)	1648[b]	303.1 (302.9)	1622[b]
H_2O	272.2 (272.4)	1661[b]	268.7 (268.9)	1655[b]	272.6 (272.8)	1628[b]	279.9 (280.1)	1608[b]

[a] Extrapolated (infinite dilution) oxygen-17 chemical shifts which were calculated assuming a monomer–dimer equilibrium and fitting the experimental data of Figure 3 (see text). The uncertainties in estimating the chemical shifts of the monomeric state were ±1.5 ppm in apolar and low dielectric constants solvents and ±0.7 ppm for high dielectric constant and protic solvents. The values in parentheses are the chemical shifts corrected for the magnetic susceptibility effects using eq 1. [b] References 41 and 42. [c] This work. [d] No IR data can be obtained due to the strong ν(CO) stretching vibration of the solvent in the same region to that of the amide I.

than the γ substituent effect observed in a variety of substituted aldehydes.[33] The difference in the chemical shift of NMA and DMA in CH_2Cl_2 and $CHCl_3$ is −5 to −6 ppm. This implies that the substituent effect of the methyl group of the amide nitrogen on the ^{17}O shielding is in the opposite direction to that of NMF. From the above it is evident that the hypothesis that *alkyl substitution of the amide nitrogen has a minor effect on $\delta(^{17}O)$ of amides is valid for the amides studied.*

The difference in the ^{17}O chemical shift of NMA and NMF in CH_2Cl_2 solution is ~3 ppm. For DMA and DMF the difference in the same solvent is 16 ppm. Methyl substitution, therefore, on the amide carbonyl carbon induces a shift of ~+3 ppm for NMF and +16 ppm for DMF. These shifts are in the opposite direction and smaller in magnitude relative to those observed upon methyl substitution at the carbonyl carbon of aldehydes (~−24 ppm). Similarly, substitution of hydrogen atoms by methyl groups in acyl derivatives introduces smaller β^{π} and γ^{π} shielding than in aldehydes and ketones.[34] This observation is in agreement with the reduced π bond order in C=O bonds of esters and amides.

(b) Effects of Solvation of the NH Group on the Amide Oxygen-17 Shielding. It has been suggested that the peptide C=O---H_2O hydrogen bond is somewhat stronger than the hydrogen bonds between water molecules and that the peptide NH---OH_2 hydrogen bond is somewhat weaker.[36] The examples of NH---OH_2 hydrogen bonds in the peptide crystals are not very frequent,[4] and a recent comprehensive analysis[9] of the overall hydration of main-chain carbonyl and amide groups in 24 high-resolution well-refined protein structures indicates that approximately 20% and 4%, respectively, of the carbonyl and amine groups that participate in secondary structure hydrogen bonds interact with the solvent. This is most likely due to the ability of the carbonyl atoms to participate in two hydrogen bonds. Further, *ab initio* calculations suggested the existence of cooperativity between the solvation of the NH and CO groups of amides.[36] It is therefore of great importance to verify the significance of the NH hydrogen bonding interaction on the amide ^{17}O shielding.

The chemical shifts of NMF, DMF, NMA, and DMA in the basic and nonprotic solvents acetone, CH_3CN, and DMSO which form a hydrogen bond with the amide hydrogen NH but not with the amide oxygen can be used to verify the significance of this hydrogen bond interaction on the amide oxygen-17 shielding. From Table 1 and taking into consideration the magnitude of the substituent effect (see discussion above) it is evident that solvation of the amide hydrogen induces a shift of ~−3 and ≤−2 ppm for NMF and NMA, respectively. It can therefore be concluded, first, that *solvation of the amide hydrogen NH induces a small modification of the oxygen-17 shielding of the amide oxygen and thus can be neglected.* Second, *cooperativity in which hydrogen bonding to NH enhances the strength of bonding to C=O is rather small.*

(c) Long Range Dipole-Dipole Interactions. Effects of the Dielectric Constant of the Medium. The extrapolated (infinite dilution) shielding constants of DMF, NMF, and DMA in *n*-hexane, CCl_4, and toluene were found to be significantly different compared with the values of 323 and 340 ppm suggested for formamides and acetamides in the absence of hydrogen bonding interactions (eqs 3 and 4). These differences can be attributed to dipole–dipole solute–solvent interactions which are a function of the dielectric constant of the medium.

The effect of a reaction field on nuclear shielding may be discussed in terms of the solvaton model.[37-39] This model does not incorporate interactions such as hydrogen bonding and protonation and thus is best suited to systems exhibiting dipole–dipole solute–solvent interactions. The solvent–solute interaction can be introduced into the paramagnetic shielding contribution σ^{p}(loc) as follows

$$\sigma^{p}(\mathrm{loc}) = -\left(\frac{\epsilon-1}{2\epsilon}\right)\frac{\mu_o e^2 h^2}{3\pi m^2}\langle r^{-3}\rangle_{2p}\sum_i\sum_k \frac{C_{\alpha k}C_{\beta k} - C_{\alpha i}C_{\beta i}}{(E_i^k - E_o)^2} \langle\psi_\alpha|H'_{solv}|\psi_\beta\rangle\,(\pi_{yz,yz} + \pi_{zx,zx} + \pi_{xy,xy}) \quad (9)$$

where μ_o, e, and m denote, respectively, the permeability of free space, the electronic charge, and mass, $\langle r^{-3}\rangle_{2p}$ is the expectation value of the inverse cube of the separation of the 2p electrons from the nucleus concerned, $E_i^k - E_o$ refers to the electronic singlet transition energy, $C_{\alpha k}$ and $C_{\beta k}$ are the matrix of linear combination coefficients of atoms A and B, respectively, ψ_α and ψ_β are the basis atomic

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Table 2. Statistics for a Least-Squares Linear Regression Analysis of $\delta(^{17}O)$ vs $(\epsilon - 1)/2\epsilon$[a]

statistics	DMF	NMF	DMA
slope	-83.2	-80.0	-80.8
corr coeff	0.95	0.96	0.93
no. of points	6	5	4
extrapolated value at $(\epsilon - 1)/2\epsilon = 0$	362.9	366.7	372.2

[a] Fitting of the chemical shift and $(\epsilon - 1)/2\epsilon$ data was performed via a linear equation of the type $y = ax + b$, where x are the $(\epsilon - 1)/2\epsilon$ values and a the slope.

orbitals employed, and the π-bond order is given by

$$\pi_{yz,yz} = 2C_{yk}C_{yi}C_{zk}C_{zi} - (C_{yi}C_{zk})^2 - (C_{zi}C_{zk})^2$$

Sum-over-states (SOS) and finite perturbation theory (FPT) calculations of aldehydes and ketones,[39] which exhibit the same solvent-dependent trends as those of amides, indicate that both sets of calculations predict increases in σ^p(loc) (decrease in $\delta(^{17}O)$) as the dielectric constant ϵ of the medium increases. An analysis of the various factors contributing to the paramagnetic term (eq 9) reveals that the increase in σ^p(loc) arises from a concomitant decrease in $\langle r^{-3}\rangle_{2p}$ which is consistent with an increase in the average separation between the ^{17}O nucleus and its 2p electrons as the charge q on the oxygen atom increases. The increase in the weighted average of the transition energies, $E_i^k - E_o$, is largely controlled by the increase in energy of the lowest $n \rightarrow \pi^*$ transition as the extent of solvation increases. Furthermore, σ^p(loc) depends on $(\epsilon - 1)/2\epsilon$, and thus, it is expected to increase drastically upon increasing ϵ for $\epsilon < 10$ but to increase rather smoothly for $\epsilon > 10$. This is in agreement with our experimental data (Table 1) which indicate that amides in CH_3CN ($\epsilon = 37.5$) and DMSO ($\epsilon = 46.7$) exhibit rather similar chemical shifts. The ^{17}O chemical shifts in *n*-hexane ($\epsilon = 1.89$), CCl_4 ($\epsilon = 2.2$), toluene ($\epsilon = 2.4$), and CH_3COCH_3 ($\epsilon = 20.7$) solution are expected to be at higher frequencies depending on ϵ.[40] This is in agreement with the experimental data. Plotting of $\delta(^{17}O)$ in the above solvents as a function of $(\epsilon - 1)/2\epsilon$ indicates a reasonably linear correlation with correlation coefficients 0.95, 0.96, and 0.93 for DMF, NMF, and DMA, respectively. (Table 2 collects the results of the least-squares analysis). In accord with the solvaton model, which does not incorporate interactions such as hydrogen bonding, the protic solvents $CHCl_3$, EtOH, MeOH, and H_2O are not included in the linear plot of Figure 4. (If the points g ($CHCl_3$), h (EtOH), i (MeOH), and j (H_2O) are included, the correlation coefficients become 0.72, 0.69, and 0.52 for DMF, NMF, and DMA, respectively.) Extrapolation at $(\epsilon - 1)/2\epsilon = 0$, which corrresponds to a hypothetical case of shielding constants in vacuo, results in the shielding constants of 362.9, 366.7, and 372.2 ppm for DMF, NMF, and DMA, respectively. These values indicate deshielding by 40–50 ppm compared to those obtained in media of high dielectric constant and demonstrate that *dipole–dipole interactions may dominate the experimentally observed ^{17}O spectral shifts of amides.*

Recently, detailed *ab initio* derivative Hartree–Fock

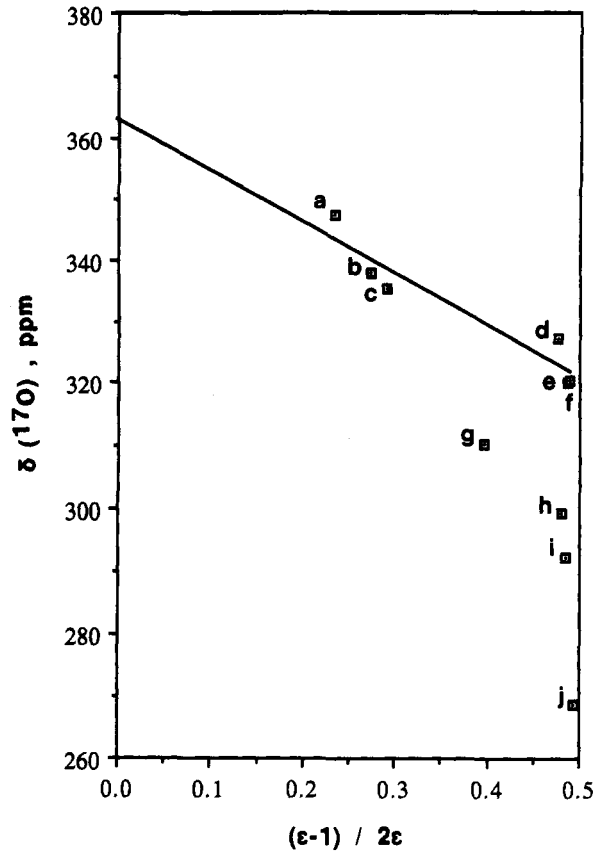


Figure 4. Dependence of $\delta(^{17}O)$ of DMF as a function of $(\epsilon - 1)/2\epsilon$, where ϵ is the dielectric constant of the solvent: a, *n*-hexane; b, CCl_4; c, toluene; d, CH_3COCH_3; e, CH_3CN; f, DMSO; g, $CHCl_3$; h, EtOH; i, MeOH; j, H_2O. Notice that the protic solvents $CHCl_3$, EtOH, MeOH and H_2O are not included in the linear plot (correlation coefficient 0.95, see Table 2).

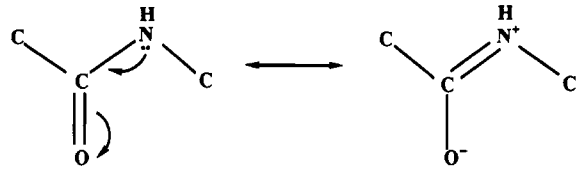
calculations were reported of the dipole and quadrupole shielding polarizabilities and hyperpolarizabilities of a number of small molecules together with estimates of the electric fields and field gradients in proteins.[41,42] These results strongly suggest that weak electrical interactions, mediated *via* the dipole and quadrupole shielding polarizabilities, can result in an increase, up to 10 ppm, in ^{17}O shielding in $C^{17}O$ labeled heme proteins (a low dielectric constant of $\epsilon = 2$ was assumed). Since the calculated dipole shielding polarizability of $C^{17}O$ (~1526.7 ppm/au field) is half that of FMA (~3195.7 ppm/au field) the increase in the ^{17}O shielding in the latter case is expected to be ~20 ppm in a medium of dielecric constant $\epsilon = 2$. This is in quantitative agreement with our experimental values of ~15–20 ppm for amides.

(d) Effects of Proton Donor Solvents. Evidence for Hydration Cooperativity Phenomena. Proton donor solvents like MeOH ($\epsilon = 32.7$) and EtOH ($\epsilon = 24.5$) induce a significant ^{17}O shielding, Table 1. This shift to low frequency may be attributed to formation of monosolvates and disolvates of the type C=O---HOR and $C{=}O(\text{---}HOR)_2$, respectively, since extensive infrared studies of the C=O stretching ν(CO) band[43,44] indicate

(40) $CHCl_3$ and CH_2Cl_2 solutions result in larger or equal shielding compared to those obtained in CH_3CN, acetone, and DMSO. It is therefore evident that nonspecific interactions between the dipole moments of the solute and solvent molecules are not the exclusive factors affecting the ^{17}O chemical shifts in CH_2Cl_2 and $CHCl_3$ solutions. Very probably, specific CH---O hydrogen bonding interactions play a significant role (Desiraju, G. R. *Acc. Chem. Res.* **1991**, *24*, 290).

(41) Augspurger, J. D.; Dykstra, C. E. *J. Phys. Chem.* **1991**, *95*, 9230.
(42) Augspurger, J.; Pearson, J. G.; Oldfield, E.; Dykstra, C. E.; Park, K. D.; Schwartz, D. *J. Magn. Reson.* **1992**, *100*, 342.
(43) Eaton, G.; Symons, M. C. R. *J. Chem. Soc., Faraday Trans.1* **1988**, *84*, 3459.
(44) Eaton, G.; Symons, M. C. R.; Rastogi, P. P. *J. Chem. Soc., Faraday Trans. 1* **1989**, *85*, 3257.

Scheme 1



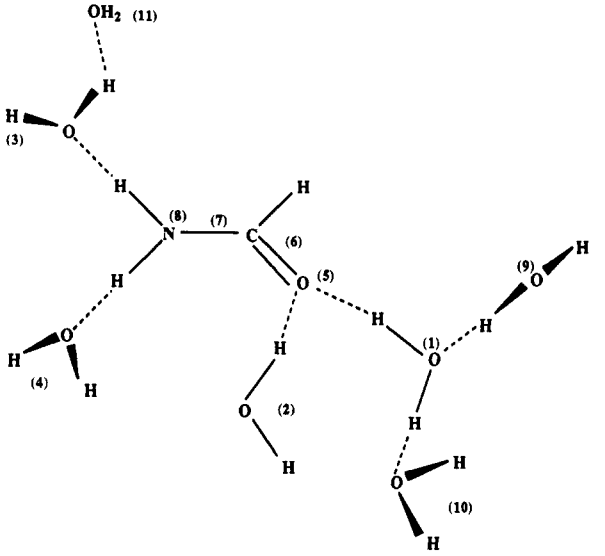
I II

that, in pure CH_3OH, NMA exists 48% in the disolvated and 52% in the monosolvated form; for DMA the respective amounts are 38% and 62%. The low-frequency shift upon hydrogen bond formation can be understood in terms of the increased contribution to form the amide resonance structure II, Scheme 1.

It has been demonstrated[28,43,44] that when amides are studied in binary protic/aprotic mixtures, discrete IR ν(CO) stretching vibration bands are formed which can tentatively been interpreted in terms of anhydrous, monosolvated, and disolvated species. In the mixed solvent D_2O/CH_3CN (~24/76 molar ratio) the amide oxygen of DMA exists essentially in the monohydrogen bonded species with ν(CO) stretching vibration shifted by 14 cm^{-1} to low frequency compared to that in pure CH_3CN. $\delta(^{17}O)$ under the same solution conditions was found to be shifted −20 ppm to low frequency compared to that which is free of specific hydrogen bonding interactions in pure CH_3CN.[45] In dilute ($<10^{-1}$ M) D_2O solution the IR spectrum exhibits only a single strong absorption due to ν(CO) stretching vibration of the dihydrated species[28,43,44] (D_2O was used in preference to H_2O in these studies because the latter has strong IR absorption bands in this spectral region). Under the same solution conditions the ^{17}O NMR signal indicates strong shielding which reflects the solvation of the amide oxygen mainly by two molecules of water (D_2O). The finding of two stable associations with the amide oxygen seems roughly in agreement with simple considerations based on lone-pair directionality. Depending on sterical constraints, the second bound molecule of water would be expected to form a weaker hydrogen bond, compared to the first bound water molecule, with the amide oxygen.[46] However, in dilute D_2O solution, a further shift to low frequency by −30 to −33 ppm was observed compared to that in the mixed solvent D_2O/CH_3CN (~24/76 molar ratio). It is therefore evident that the contribution of specific hydrogen bonds of the bound molecules of water at the amide oxygen is cooperative, presumably due to increased dielectric constant of the medium and further solvation of the bound molecules of water.

The decrease of $\delta(^{17}O)$ as the extent of the dielectric constant of the medium increases is in apparent agreement with the previous discussion on the effect of a reaction field on ^{17}O nuclear shielding. Furthermore, it demonstrates that the energy of hydrogen bond interaction increases as the dielectric constant of the medium increases. This is the opposite of what is expected in the case of the electrostatic interaction between ions

Chart 1



$$E = -\frac{q_i q_j}{\epsilon r_{ij}} \quad (10)$$

where q_i and q_j are point charges and r_{ij} is the interatomic distance between atoms i and j or in the case of the dipole interaction between bond dipoles μ_{ij}[47]

$$E = -\mu_{ij}\mu_{ij}[2\cos\theta_i\cos\theta_j - \sin\theta_i\sin\theta_j\cos\alpha]/4\pi\epsilon r_{ij}^3 \quad (11)$$

where α is the angle between the dipoles and θ_i and θ_j are the angles between the dipoles and r_{ij} where r_{ij} is the distance between the midpoints of the bonds. Evidently, the energy of the electrostatic interaction involving dipoles, such as the amide and water dipoles, is more complex than that denoted by eq 11 because the extent of the dipolar nature of such molecules is often changed, *i.e.*, induced by the other molecules with which it is interacting.

Cooperativity in hydrogen bonds has been investigated theoretically in a variety of molecular systems.[48] Johansson *et al.*[36] investigated nonadditivity solvation phenomena in amides and whether the presence of the first water molecule affects the ability of the second water to form a hydrogen bond either to an amide or to a water bonded to an amide. In the cases where the central water molecule is functioning as a proton donor and acceptor (1) + (9) and (3) + (11) (Chart 1) the net hydrogen bonding is stronger (−1.7 and −1.6 kcal/mol, respectively) than one would expect from adding the H-bond energies of water (1) − amide, water (1) − water (9), and the small attraction of water (9) − amide. In this case cooperativity in hydrogen bonding is positive. When the central water is functioning as a double proton donor, the cooperativity is negative (anticooperativity), 1.6 kcal/mol, indicating that the amide–$2H_2O$ complex is less tightly bound than one would expect by adding up the two-body energies. The present ^{17}O NMR results, therefore, provide *experimental evidence for cooperativity and nonadditive contribution of hydrogen bonds at the amide oxygen.* Clearly, further experimental and theoretical studies of cooperative hy-

(45) A decrease in both $\delta(^{17}O)$ and ν(CO) was also observed in $C^{17}O$ labeled heme proteins upon interaction with $X^{\delta+}$, which could be a hydrogen bond donor, a charged species such as an imidazolium residue (histidine), or possibly an alkylammonium ion (lysine) or a guanidinium residue (arginine) (Park, K. D.; Guo, K.; Adebodun, F.; Chiu, M. L.; Sligar, S. G.; Oldfield, E. *Biochemistry* **1991**, *30*, 2333).

(46) Del Bene, J. E. *J. Am. Chem. Soc.* **1978**, *100*, 1387.

(47) Israelachvili, J. *Intermolecular & Surface Forces*, 2nd ed.; Academic Press: New York, 1992.

(48) Finney, J. L. In *Structure and Dynamics in Biological Systems*, Jonsson, B., Ed.; Cambridge University Press: Cambridge, 1989, pp 123–130.

drogen bonding of amide–$(H_2O)_n$ systems seems to be worthwhile.

(e) On the Significance of Wave Function Overlap in Hydrogen Bonding Interactions. There has been some controversy as to whether wave function overlap (covalency) contributes significantly to the hydrogen bond.[49–51] In cases of very strong hydrogen bonds, such as in the bifluoride $[F{\cdots}H{-}F]^-$ ion, it is certain that wave function overlap makes a significant contribution to the hydrogen bond energy. In peptides and proteins, however, distance reductions and binding energies are much smaller and thus the contribution of wave function overlap is considered insignificant. Further, many recently developed molecular mechanics programs tend to simplify the hydrogen bond and do not include functions specifically for hydrogen bond interactions. It is considered that hydrogen bonding is adequately accounted for by the electrostatic and nonbonding Lennard–Jones terms, *i.e.*, 9-6-1 or 12-6-1 potentials. As discussed in detail in section c specific influences which arise from electronic interactions between the dipole moments of the solute and solvent molecules result in changes in the σ^p(loc) that are proportional to $(\epsilon - 1)/2\epsilon$. As previously emphasized, plotting of $\delta(^{17}O)$, in nonprotic solvents, as a function of $(\epsilon - 1)/2\epsilon$ indicates a linear correlation. Protic solvents result in significantly larger shifts to low frequencies which strongly deviate from the $\delta(^{17}O)$ *vs* $(\epsilon - 1)/2\epsilon$ correlation (Figure 4). It is therefore evident that specific dipole–dipole interactions between the solute and solvent molecules are not the exclusive factors affecting the ^{17}O chemical shifts. Very probably, the contribution of wave function overlap, *i.e.*, covalent charge transfer bonded interaction, is not insignificant. Consequently, the structure of the hydrated species is, contrary to pure dipole–dipole interactions, not determined by interactions between the overall dipoles but merely by the interactions of lone pairs.

(f) Comparison with ab Initio Calculations. *Ab initio* calculations using gauge invariant atomic orbitals (GIAO s) within the FPT framework have been reported for FMA in the presence of its first hydration shell.[52] The perturbation treatments as well as the determination of the unperturbed SCF wavefunctions were carried out using Gaussian basis functions. Most of the results were obtained with the minimal basis set and, in addition, some of them with a split basis for the valence shell. With the exception of the formyl proton, which is not involved in hydrogen bonding, significant variations in the shielding of the formamide nuclei are produced by hydration (Figure 5). The values reported for the complexes FMA–$(H_2O)_2$ show that the shift observed when the oxygen atom is directly involved in the hydrogen bond is much larger than the one occurring when the water molecules are bound to the NH bonds. The variation was calculated to be to low frequency in both cases. This is in agreement with ^{17}O NMR experimental data on formamide[21] and several amides in different solvents (Table 1). It was subsequently noticed that the shift to low frequency when going from acetone to water (~53 ppm) is significantly smaller than

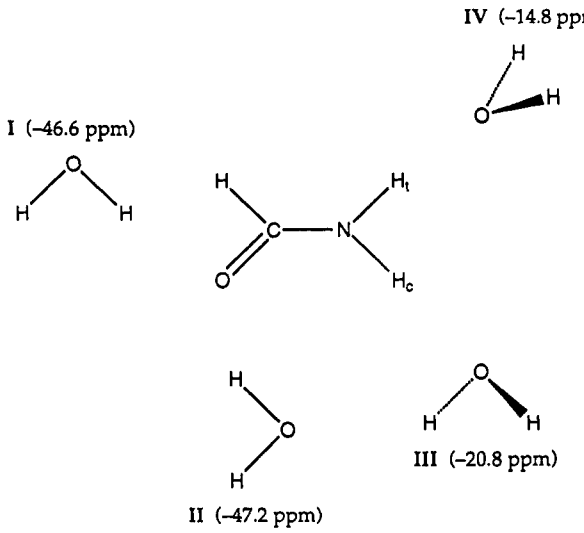


Figure 5. Arrangement of bound molecules of H_2O with respect to FMA. The values in parentheses are calculated shielding changes[52] due to a particular hydrogen bond.

Table 3. Statistics for a Least-Squares Linear Regression Analysis of $\delta(^{17}O)$ vs ν(CO) of the Data of Table 1[a]

statistics	DMF	NMF	DMA	NMA
slope	1.78	1.32	1.95	1.19
corr coeff	0.968	0.973	0.978	0.985
no. of points	9	8	7	5
std dev of regression	6.53	5.95	5.61	5.09

[a] Fitting of the chemical shift and stretching frequency data was performed via a linear equation of the type $y = ax + b$, where x are the ν(CO) values and a the slope.

the one calculated between FMA–$(H_2O)_2$ III + IV and FMA–$(H_2O)_4$ (93 ppm). In our opinion, the results obtained with the minimal basis set are in quantitative agreement with the experiment and do not overestimate the effect of water–carbonyl hydrogen bonds on the ^{17}O chemical shift when a proper comparison is made with the shielding constants of amides in vacuum rather than in acetone. Indeed, the extrapolated $(\epsilon - 1)/2\epsilon = 0$ values for DMF, NMF, and DMA where found to be at ~+95 ppm compared to those in aqueous solution and in excellent agreement with the value of +93 ppm for the hydrated FMA derived from *ab initio* calculations.

2. Correlation between $\delta(^{17}O)$ and ν(CO). Plotting of the ^{17}O chemical shift of DMF, DMA, NMF, and NMA *vs* ν(CO) stretching vibration in various solvents which have varying dielectric constants and solvation abilities indicates an excellent correlation (Figure 6). Table 3 collects the results of the least-squares analysis. The resulting correlation demonstrates that both $\delta(^{17}O)$ and ν(CO) appear to be reflecting a similar type of electronic perturbation due to hydrogen bonding and dipole-dipole interactions.[53,54] However, $\delta(^{17}O)$ appears to be more sensitive to electronic perturbations, compared to ν(CO), as reflected by the values of the slopes of the resulting correlations (Table 3). For solutions in alcohols, two

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(53) Good linear correlations between $\delta(^{17}O)$ and ν(CO) were also observed in a number of $C^{17}O$ labeled heme proteins.[45] Further, *ab initio* calculations demonstrated an essentially linear correlation between the vibrational frequency of carbon monoxide and its $\delta(^{17}O)$ under a variety of external electrical potential, *i.e.*, various uniform electric fields, field gradients, and various positions of an axial dipole consisting of two point charges (Augspurger, J. D.; Dykstra, C. E.; Oldfield, E. *J. Am. Chem. Soc.* **1991**, *113*, 2447).
(54) This linear relationship may prove useful for the estimation of ^{17}O chemical shifts from more readily available IR data.

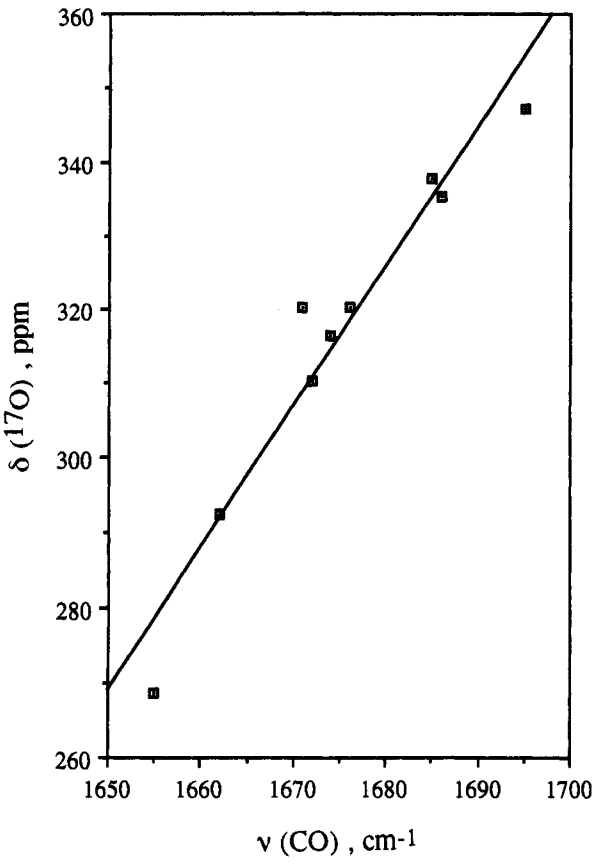

Figure 6. Graphical relationship between infrared CO vibrational stretching frequency [ν(CO), cm^{-1}] and ^{17}O NMR isotropic chemical shift [$\delta(^{17}O)$, ppm] for DMF.

infrared bands were detected due to an equilibrium of two species (see discussion above). In these cases weighted mean shifts were calculated, and these were used in the correlation of Figure 6. This suggests that, on the NMR time scale, the various solvation species are in fast exchange and only contribute to time-averaged features. This contrasts to vibrational spectroscopy in which the time scale is too short for many averaging processes; hence, discrete solvates can be detected due to non-hydrogen bonded, monosolvated, and disolvated species.

The observed vibrational shifts due to solvation phenomena were found to vary significantly among the amides studied. Thus, for DMF the differential shift between *n*-hexane and water is 40 cm^{-1} and between CH_3CN and D_2O 21 cm^{-1}, while for DMA the respective changes are 64 and 36 cm^{-1}. The cause of these differences is still unclear. On the contrary, the ^{17}O shielding changes due to dipole–dipole interactions and specific hydration at the amide oxygen are practically independent of *N*-methyl and amide carbon substitution. Thus, the hydration state of more complex peptides can be deduced by comparison with the spectroscopic characteristics of simple amides.

Conclusions

From the data presented here the following can be concluded. (i) The methyl substitution of the amide nitrogen induces an ^{17}O shift of +4 to +6 ppm and −5 to −6 ppm for NMF and NMA, respectively, and thus can be neglected. (ii) Solvation of the amide NH group induces a shift of the amide oxygen to low frequency by ≤−3 ppm for both NMF and DMF. This is very small compared to the overall chemical shift changes of the amide oxygen due to solvation phenomena and thus can be neglected. (iii) Long range dipole–dipole interactions, specific hydration at the amide oxygen, and cooperativity in hydrogen bonding of the bound molecules of water can introduce significant shielding of the ^{17}O nucleus. Thus, the overall chemical shift change between an amide oxygen in the absence of hydrogen bonding interactions in vacuum and that which is fully hydrated in aqueous solution is not 50–55 ppm, as originally suggested, but over 95 ppm. This significant shift is, in part, due to the covalent character of the hydrogen bonding interaction. (iv) Linear correlation between $\delta(^{17}O)$ and ν(CO) exists for different solvents which have varying dielectric constants and solvation abilities. This demonstrates that both IR and ^{17}O NMR spectroscopy appear to be reflecting a similar type of electronic perturbation, *i.e.*, hydrogen bonding and dipole–dipole solute–solvent interactions.

The great sensitivity, therefore, of the ^{17}O shielding constants to both long-range dipole–dipole interactions and specific hydrogen bonding interactions at the amide oxygen might prove a valuable, complementary tool to IR spectroscopy in studying specific hydration phenomena of amides and peptides in solution.

Acknowledgment. This work was supported by the Research Committee of the University of Ioannina and the Greek General Secretary of Research and Technology. We appreciated useful comments and suggestions from the referees.

Abbreviations: DMA, dimethyl acetamide; DMF, dimethyl formamide; FMA, formamide; NMA, *N*-methylacetamide; GIAOs, gauge, invariant atomic orbitals; NMF, *N*-methylformamide; FPT, finite perturbation theory; SCF, self-consistent field.