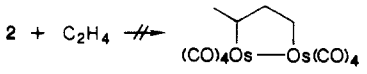
by Keim and co-workers[34] after they discovered that the carbonyl ligands of **7** were susceptible to attack by methoxide.

Finally, we have compared the reactivity of **2** toward C_2H_4 with its reactivity toward CO. Whereas **1** and C_2H_4 form a diosmacyclopentane,[3b] **8** is not formed when **2** is heated under 1 atm of ethylene.[35] Alkyl substitution on the carbon of a diosmacyclopropane thus affects its reactivity toward CO insertion and toward C_2H_4 in quite different ways.



(9)

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Acknowledgment. We are grateful to the Department of Energy for support under Grant DE-FG02-84ER13299.A005 and to Bruce Bender for assistance in the preparation of this manuscript.

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(35) However, ^{1}H NMR (δ 1.75 in C_6D_6, d, J = 2.3 Hz, $CHCH_3$) and IR (2121, 2076, 2040, 2032, 2020, 2012, and 1994 cm^{-1}) spectra indicated that **8** had been formed by the reaction between $TfOCH_2CH_2CH(CH_3)OTf$ and $Na_2[Os_2CO)_8]$. None of the butenes produced by the thermal decomposition (4 h at 81 °C and 3.5 h at 114 °C, in C_6D_6) of **8** thus prepared were seen when a solution of **2** was treated with an atm of C_2H_4 under the same conditions—an observation which eliminated the possibility that **8** was formed in reaction 9 but decomposed.

Clean TOCSY for ^{1}H Spin System Identification in Macromolecules

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Received March 21, 1988

Two-dimensional (2D) correlation spectroscopy (COSY)[1-3] and 2D nuclear Overhauser spectroscopy (NOESY)[4,5] provided the experimental basis that made nuclear magnetic resonance (NMR) an efficient method for the determination of three-dimensional protein structures in solution.[6,7] Recently, these two basic experiments were complemented by 2D experiments in the rotating frame which involve an extended mixing period in the presence of radio frequency irradiation. Total correlation spectroscopy (TOCSY,[8] sometimes called HOHAHA[9]) gives multistep COSY-type information. By virtue of the in-phase cross-peak multiplet structure, which contrasts with the antiphase structure in COSY spectra, TOCSY peaks are often stronger and hence easier to detect than the corresponding COSY peaks. Rotating frame cross-relaxation spectroscopy (CAMELSPIN,[10] here called ROESY[11]) is an alternative to NOESY where cross-peak intensity depends differently on molecular correlation times.[10]

Rotating frame experiments produce simultaneously coherence transfer through J coupling and through cross relaxation, which may lead to undesired interference and ambiguities between TOCSY and ROESY effects. In this communication we introduce a technique that provides, for the first time, clean TOCSY spectra and suppresses contributions from cross relaxation based on the opposite signs of longitudinal and transverse cross-relaxation rates in large molecules.[7,10]

A conventional TOCSY spectrum of the N-terminal domain 1-76 of the protein P22 c2 repressor is shown in Figure 1 (parts A and B). Apart from the positive peaks due to J transfer, it contains also negative peaks due to cross relaxation. Ambiguities arise whenever peaks of different sign coincide. A clean TOCSY spectrum of the same molecule, recorded with the modified technique to be described in the following, is given in Figure 1 (parts C and D). The absence of negative peaks is readily apparent, in particular in the regions containing cross peaks between different amide protons and between amide and α- or β-protons.

Cross relaxation between two nuclei i and k at specified chemical shifts is determined by the rate constant $\Gamma_{ik} = (1/t_c) \int_0^{t_c} \boldsymbol{n}_i(t) \boldsymbol{\Gamma} \boldsymbol{n}_k(t)\, dt$.[12] $\boldsymbol{\Gamma}$ is the diagonal relaxation matrix with the elements (Γ^{tcr}, Γ^{tcr}, Γ^{lcr}). Γ^{tcr} denotes the rate constant for cross relaxation of transverse components, while Γ^{lcr} applies to longitudinal components; t_c is the duration of one pulse cycle. The time-dependent vectors $\boldsymbol{n}_i(t)$ and $\boldsymbol{n}_k(t)$ are the so-called *invariant trajectories* of the two spins that depend on their chemical shifts and on the pulse sequence. Each is characterized by the unique property that a magnetization vector travelling on it during a multiple pulse sequence returns periodically to its origin.

In the case of an MLEV-17 pulse sequence[9,13] with the composite pulse $R = 90^\circ_x\, 180^\circ_y\, 90^\circ_x$ without intervals between the pulses, the invariant trajectory for on-resonance conditions starts along the y-axis, rotates during the first pulse to the z-axis, during the second pulse to the $(-z)$-axis, and during the third pulse back to the y-axis. The above expression for Γ_{ik} leads to the cross-relaxation rate constant $\Gamma_{ik} = {}^1/_2(\Gamma^{lcr} + \Gamma^{tcr})$, as the two trajectories $\boldsymbol{n}_i(t)$ and $\boldsymbol{n}_k(t)$ spend equal time in the longitudinal and transverse directions. In the case of nonideal pulses due to rf field inhomogeneity and off-resonance conditions, the two relaxation rate constants are differently weighted: $\Gamma_{ik} = (1 + \lambda)^{-1} (\Gamma^{lcr} + \lambda\Gamma^{tcr})$, where λ depends on the distribution of the effective pulse angles and on off-resonance frequencies. Trim pulses are applied before and after the MLEV-17 sequence to destroy all magnetization components that are not aligned along the initial and final orientation of the invariant trajectory. The simulated cross-relaxation rate constant under the standard MLEV-17 pulse sequence is plotted as a function of the two offset frequencies in Figure 2A. The calculation was based on the experimentally determined rf field distribution in our probe. Significant ROESY cross peaks are found in agreement with Figure 1 (parts A and B).

Cross relaxation can be suppressed when the effects of longitudinal and transverse relaxation are forced to compensate each other. This is possible by the introduction of two delays Δ before and after the 180°_y pulse in the MLEV-17 sequence, leading to the composite pulse $R' = 90_x^\circ - \Delta - 180_y^\circ - \Delta - 90_x^\circ$. The modified cross-relaxation rate constant on resonance is now $\Gamma_{ik} = (2 + r)^{-1}[\Gamma^{tcr} + (1 + r)\Gamma^{lcr}]$ with $r = \Delta/\tau_{90}$ (τ_{90} is the duration of a 90° pulse). Thus, cross relaxation is suppressed when $r = -1 - \Gamma^{tcr}/\Gamma^{lcr}$. This equation has a physical solution when Γ^{lcr} is negative, i.e., for bulky molecules with $\omega_0\tau_c > \sqrt{5}/2$ (ω_0 = res-

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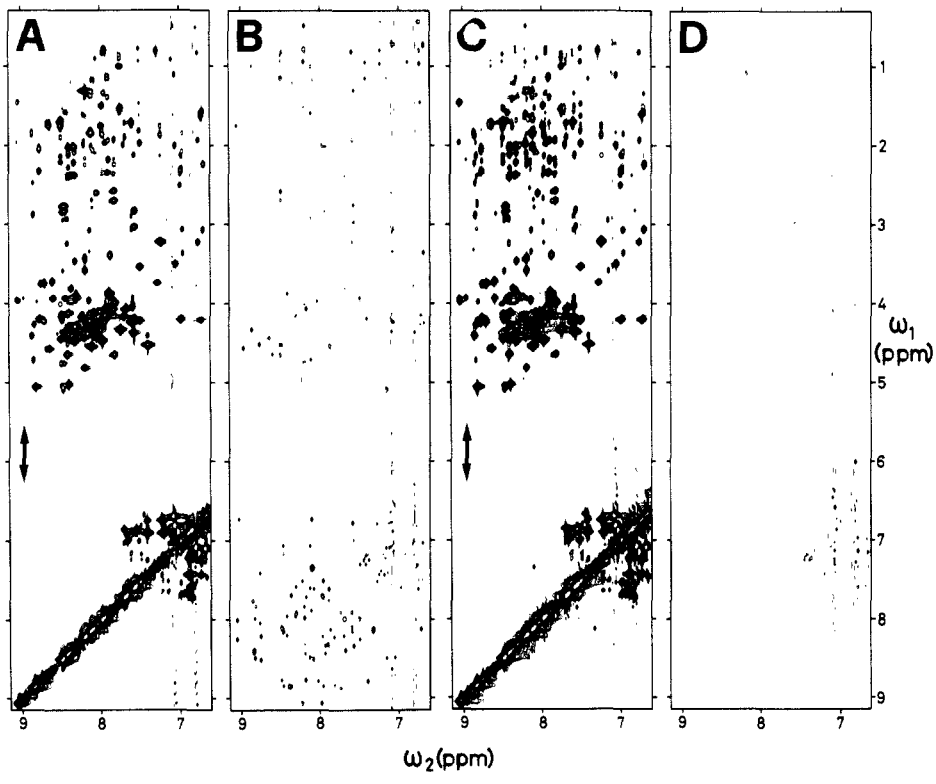

Figure 1. Phase-sensitive ^1H TOCSY spectra of 5 mM P22 c2 repressor 1-76[14] in 90% H_2O/10% D_2O, pH 6.0, 28 °C, recorded at 600 MHz on a Bruker AM600 spectrometer with a 60 ms mixing time including two trim pulses of 2 ms duration before and after the MLEV-17 mixing. A delay of 2 μs was introduced between all pulses to allow for phase shifting. The spectral region shown contains the diagonal peaks of the amide protons and all cross peaks between nonlabile protons and amide protons. (A) Mixing with conventional MLEV-17, τ_{90} = 27 μs, t_c = 1.8 ms, 17th pulse: β = 60°. Only positive levels are shown. (B) Same as (A), but only negative levels are plotted. (C) Clean TOCSY spectrum obtained by mixing with a two-window MLEV-17 sequence, τ_{90} = 12.2 μs, Δ = 31.9 μs, t_c = 1.8 ms. Positive levels. (D) Negative levels of the spectrum of Figure 1C. The remaining negative peaks are from t_1-noise and from antiphase contributions to narrow cross peaks.

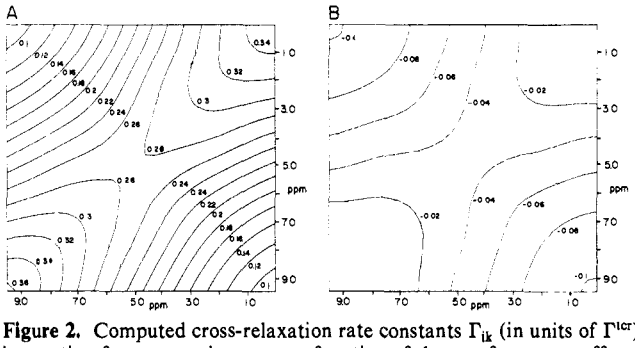

Figure 2. Computed cross-relaxation rate constants Γ_{ik} (in units of Γ^{tcr}) in rotating frame experiments as a function of the two frequency offsets for the experimentally determined rf field distribution, a spectral width of 5.7 kHz, and identical pulse durations and delays as in the experiments in Figure 1. $\Gamma^{tcr}/\Gamma^{lcr} = -2$ is assumed: (A) TOCSY using the conventional MLEV-17 sequence and (B) clean TOCSY using a two-window MLEV-17 sequence with r = 2.6.

onance frequency, τ_c = rotational correlation time). The shortest delay Δ is reached for $\tau_c \rightarrow \infty$ where $\Gamma^{tcr} = -2\Gamma^{lcr}$, yielding r = 1 or Δ = τ_{90}.

The value r = 1 expected to be optimum for the P22 c2 repressor 1-76 with a correlation time of $\tau_c \approx 6$ ns led to incomplete suppression of cross-relaxation peaks in the modified TOCSY experiment. Excellent suppression could however be achieved, as shown in Figure 1 (parts C and D), with r = 2.6 which was calculated based on observed pure cross relaxation peak intensities $I^{cross}(r)$ in two TOCSY spectra with MLEV-17 (r = 0) and two-window MLEV-17 (r = 1), respectively, using the linear extrapolation $r_{opt} = (I^{cross}(0))/(I^{cross}(0) - I^{cross}(1))$. The optimum value r = 2.6 can be explained by rf inhomogeneities of the probe. A computation of the cross-relaxation rate constant under the same conditions as for Figure 2A but with a two-window MLEV-17 sequence with r = 2.6, is shown in Figure 2B. It exemplifies efficient suppression of cross relaxation in agreement with the

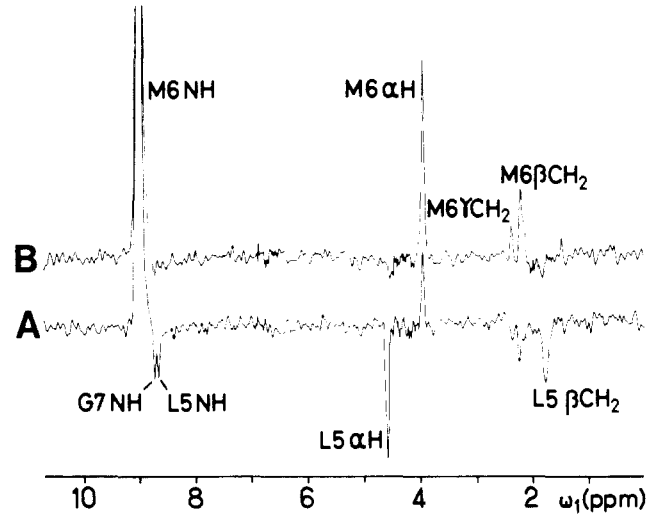

Figure 3. Sections parallel to the ω_1-axis through the NH diagonal peak of Met-6 at 8.96 ppm (see arrows in Figure 1 (parts A and C)). The resonances are identified with the one-letter amino acid symbol and their sequence number in the polypeptide chain: (A) conventional TOCSY and (B) clean TOCSY.

experimental spectrum of Figure 1 (parts C and D). Sections through the experimental spectra of Figure 1, shown in Figure 3, demonstrate that in the conventional experiment cross relaxation dominates J transfer contributions for peaks such as NH-βCH_2 and NH-γCH_2 of methionine-6, while for the modified experiment with the two-window MLEV-17 sequence the J cross peaks are clearly apparent. In addition, the cross-relaxation peaks between Met-6NH and protons from other amino acid residues are strongly suppressed.

In conclusion, the introduction of delays into an MLEV-17 pulse sequence allows the suppression of cross relaxation in TOCSY

under practical, experimental conditions with nonideal pulses. With this modification the TOCSY experiment carries the promise to facilitate ^{1}H spin system identifications in macromolecules and to enable such studies with bigger molecules than would be possible with presently available techniques.

Acknowledgment. This research has been supported by the Swiss National Science Foundation and by the Kommission zur Förderung der Wissenschaftlichen Forschung. The manuscript has been processed by I. Müller.

Hg_{18}–Metallothionein

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We report the formation of a novel mercury–protein complex, namely Hg_{18}–metallothionein, from rabbit liver metallothionein (MT) isoform 2. This new species is characterized by a strong circular dichroism (CD) intensity under the thiolate-to-mercury charge-transfer bands in the 240–360 nm region. The presence of this unusually intense CD spectrum suggests that Hg_{18}-MT 2 adopts a specific 3-dimensional structure not found previously for MT species, rather than the random coil structure expected for such a high metal loading.

Metallothioneins (MT) are low molecular weight, cysteine-rich proteins containing 20 SH groups per molecule.[1] MT binds a wide range of metal ions both in vivo and in vitro.[2,3] The stoichiometric ratio for the sum of Zn and Cd binding to MT is 7,[3] while for Cu[3-5] and Ag[3] the ratio is 12. Few spectroscopic, structural and stoichiometric data are available for Hg,[3,6-9] despite the importance of this element in metal toxicity. The only species reported to date for Hg is Hg_7-MT.[3,6,7]

CD spectral intensity with group 11 and 12 metals bound to metallothionein arises from ligand-to-metal charge transfer (LMCT).[4,6,10] CD spectra probe the chirality of the whole metal binding site cage. The absence of aromatic amino acids results in a spectral window in the wavelength region of these charge-transfer transitions.[4,10]

Figure 1 shows that isodichroic formation of Hg_7-MT[11] takes place when Hg is added to apo-MT 2 at pH 2.4, resulting in band maxima at 310 nm (+) and 270 nm (−), and the presence of a symmetrical well in intensity between 260 and 290 nm in the contour level diagram. The magnetic circular dichroism (MCD) spectrum suggests that Hg_7-MT formed at pH 2.4[14] does not adopt a geometry similar to that of Cd_7-MT.[15,16]

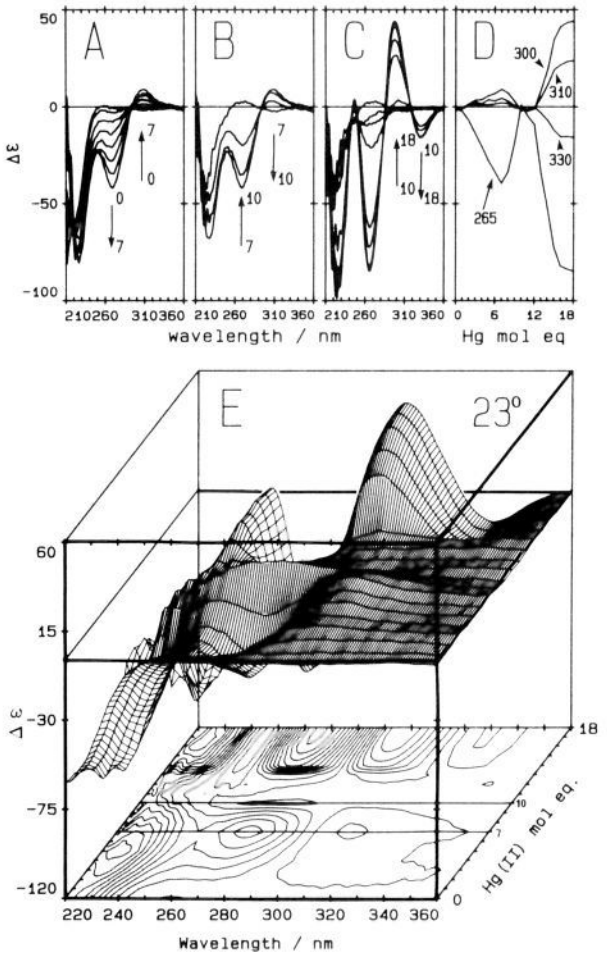

Figure 1. CD spectra recorded during a titration of rabbit liver apo-MT 2 with Hg(II) at pH 2.4. (A) 0–7, (B) 7–10, (C) 10–18 mol equiv of Hg; (D) Intensities as a function of mol equiv of Hg; (E) 0–18 mol equiv, the third axis in (E) is mol equiv of Hg.

As more Hg is added (up to 10 mol equiv), the Hg_7-MT CD signal diminishes as a new, but weaker, signal forms isodichroically (Figure 1B). Surprisingly, once 12 mol equiv of Hg(II) have been added, a very strong CD spectrum begins to form isodichroically (Figure 1C), reaching a maximum intensity at 18 mol equiv. Figure 1D shows changes in intensity as a function of mol equiv of Hg added at the band maxima for Hg_{18}-MT 2 (300 (+), 330 (−), 265 nm (−)) and also at 310 nm (band maximum (+) for Hg_7-MT). No further changes are found with up to 40 mol equiv of Hg. The complex is stable between pH 2 and 6.9; the CD spectral envelope collapses above pH 7.

A new Hg_7-MT species forms at 2 °C and pH 2.4 (Figure 2) unlike Cd_7-MT which does not form below 5 °C.[17] However, significantly, only a small fraction of the Hg_{18}-MT expected forms as more Hg is added. Subsequent warming to 37 °C for 10 min results in complete formation of Hg_{18}-MT (Figure 2C). The

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