It is shown in Figure 5 along with conformation 8.

Our results may be compared with both ab initio results and results recently published by Smith and co-workers.[10] Ab initio calculations in fact suggest preferences for the Φ, Ψ combination of 50°, 0° that arises as the preferred geometry in our results.[24] This enhances confidence in our work in the ab initio calculations. Using phospholipid bilayers oriented between glass plates, Smith and co-workers, investigated the conformational properties of a deuterated 1,2-di-*O*-tetradecyl-3-*O*-(β-D-glucopyranosyl)glycerol. The conformations they prefer are similar to ours in several respects. The sugar head group was found to be extended away from the bilayer surface. The angle between the C1–O1 bond and the acyl chain director, which is related to Ψ, lies between 156 and 173°. The corresponding angle in conformation 1 is calculated to be 126°. Smith and co-workers are not able to settle on one value for the angle corresponding to Φ out of the four possibilities they list, though they tend to favor the lower values of Φ. Our value of Φ would be 60°. The glycolipids studied are, of course, not identical. One difference is in the nature of the monosaccharide, glucose in Smith's glycolipid and galactose in ours. The two monosaccharides are different in the configuration at C4, where galactose has an axial hydroxyl instead of an equatorial hydroxyl group. This difference is spectroscopically useful, because in galactose the C–D bond at the 4 position is not collinear with the C–D bond at 3, thus yielding an additional piece of conformational information. The different hydroxyl orientation may also be in part responsible for the structural variations observed.

The other difference in DLAM is the existence of a hydrophilic spacer region. The presence of a hydrophilic spacer region would imply an increased level of flexibility compared to a direct attachment of the sugars to the glycerol backbone. Implicit in our treatment is the assumption of a single-order parameter scaling down all the deuterium splittings and the assumption of overall rigidity of the molecule. This assumption of molecular rigidity is certainly very valid in the sugar ring region but it is less likely to be so in the hydrophilic spacer region. Variations of the deuterium splittings with temperature provide one test of this assumption. If separate order tensors exist for the ring and spacer region, they are likely to have different temperature dependencies. Splittings for H8, H4, and H3 scale identically as a function of temperature. This adds credence to the use of a single-order parameter to scale both ring C–D vectors and the C–D vector in the hydrophilic spacer region.

It may be possible in the future to relax these assumptions by inclusion of more data, perhaps from proton–dipolar splittings or through-bond scalar coupling. For the time being, a methodology has been presented that combines experimentally derived information, in the form of deuterium NMR quadrupolar splittings with a molecular mechanics program, to arrive at a reasonable conformation for glycolipid head group in a membrane-like environment. This provides a useful means of investigating properties of this very important class of molecules.

Acknowledgment. We thank J. N. Scarsdale for his advice on the implementation of the pseudoenergy potential and L. E. Kay for his assistance in the proton simulations. This research was supported by grants from the National Institutes of Health (GM 191035 and GM 33225), and benefited from instrumentation provided through shared instrumentation program of the Division of Research Resources of the National Institutes of Health (RR 02379).

Registry No. DLAM, 69313-68-4.

(24) Jeffrey, G. A.; Pople, J. A.; Radom, L. *Carbohydr. Res.* **1972**, *25*, 117–131.

Heteronuclear Filters for Two-Dimensional ^{1}H NMR. Identification of the Metal-Bound Amino Acids in Metallothionein and Observation of Small Heteronuclear Long-Range Couplings[1]

Erich Wörgötter,[†,§] Gerhard Wagner,[†,∥] Milan Vašák,[‡] Jeremias H. R. Kägi,[‡] and Kurt Wüthrich*[†]

Contribution from the Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule-Hönggerberg, CH-8093 Zürich, Switzerland, and Biochemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8093 Zürich, Switzerland.
Received July 20, 1987

Abstract: The use of heteronuclear filters for editing of two-dimensional homonuclear ^{1}H NMR experiments is illustrated with studies of [$^{113}Cd_7$]-metallothionein. The previously described X-filter and X(ω_2)-half-filter are employed with an improved phase-cycling scheme to identify the amino acid residues bound to ^{113}Cd. The paper describes the first realization of a 2X-filter, which selects for protons with nonvanishing spin–spin couplings to two X spins and thus presents a new criterion for direct identification of bridging cysteines in metallothioneins. It is further demonstrated that two-dimensional ^{1}H correlation experiments (COSY) recorded with heteronuclear filters enable the identification of small heteronuclear long-range spin–spin couplings, which are otherwise difficult to observe.

During the period 1979–1982, the method of sequential resonance assignments in biopolymers was introduced for proteins[2] and subsequently adapted also for nucleic acids.[3] This technique can rely entirely on homonuclear ^{1}H NMR, provided that sufficiently well-resolved ^{1}H NMR spectra are available.[4] For a variety of small proteins the sequential assignment method ef-

† Eidgenössische Technische Hochschule-Hönggerberg.
‡ Universität Zürich.
§ Present address: Biochemie GmbH, A-6250 Kundl, Austria.
∥ Present address: Institute of Science and Technology, Biophysics Research Division, University of Michigan, Ann Arbor, MI 48109.

(1) Abbreviations used: NMR, nuclear magnetic resonance; ppm, parts per million; 2D, two-dimensional; COSY, 2D correlated spectroscopy; TOCSY, 2D total correlation spectroscopy; NOESY, 2D nuclear Overhauser and exchange spectroscopy; RELAYED-COSY, 2D relayed coherence transfer spectroscopy; E.COSY, 2D exclusive correlated spectroscopy; MT2, metallothionein isoprotein 2 from rat liver; TSP, (trimethylsilyl)propionic acid sodium salt.

0002-7863/88/1510-2388$01.50/0

Table I. Survey of Heteronuclear Filters for Editing of Homonuclear 2D ^1H NMR Experiments with the Four-Spin System ^1H, $^1H'$, X, and X'

order of selected coherence	heteronuclear filter[7,8]	condns for obsvn of: diagonal peaks ^1H	diagonal peaks $^1H'$	cross peaks[a] (^1H,$^1H'$)	cross peaks[a] ($^1H'$,^1H)
2(HX)	X-filter	a	b	c	c
	X(ω_2)-half-filter	a	b	d	e
	X(ω_1)-half-filter	a	b	e	d
3(HXX')	2X-filter	f	g	h	h
	2X(ω_2)-half-filter	f	g	i	k
	2X(ω_1)-half-filter	f	g	k	i

[a] (^1H,$^1H'$) is a cross peak with the resonance frequencies of ^1H and $^1H'$ along ω_1 and ω_2, respectively, and ($^1H'$,^1H), vice versa. The letters indicate that the following spin–spin coupling constants must be non-vanishing ($J \neq 0$): a, J_{HX}; b, $J_{H'X}$; c, J_{HX}, $J_{H'X}$, $J_{HH'}$ (or $J_{HX'}$, $J_{H'X'}$, $J_{HH'}$); d, $J_{H'X}$, $J_{HH'}$; e, J_{HX}, $J_{HH'}$; f, J_{HX}, $J_{HX'}$; g, $J_{H'X}$, $J_{H'X'}$; h, J_{HX}, $J_{HX'}$, $J_{H'X}$, $J_{H'X'}$, $J_{HH'}$; i, $J_{H'X}$, $J_{H'X'}$, $J_{HH'}$; k, J_{HX}, $J_{HX'}$, $J_{HH'}$.

ficiently provided sequence-specific ^1H NMR assignments, enabling for the first time the determination of the three-dimensional structure of noncrystalline proteins.[5] Limitations of the method arise if the ^1H NMR spectra are incompletely resolved in 2D NMR experiments. Quite naturally this is expected for big molecules, which contain a large number of resonance lines, but for a variety of reasons incomplete spectral resolution may also be encountered in small proteins.[4] As was pointed out by several research groups,[6] isotope labeling, e.g., with ^{13}C or ^{15}N, is an alternative approach for obtaining ^1H NMR assignments, which can work also in quite high molecular weight systems. For practical reasons sequence-specific resonance assignments by site-specific isotope labeling will probably always be limited in scope. The combination of residue-specific labeling with sequential assignment techniques, however, carries great promise for extending detailed NMR studies of molecular structures and intermolecular interactions to more complex systems.[4] For the purpose of such investigations, we recently proposed different heteronuclear filtering techniques for editing 2D ^1H NMR spectra, e.g., COSY, TOCSY and NOESY, with respect to heteronuclear scalar couplings J_{HX}.[7,8] In this paper we report on further progress with this class of experiments. This includes the first experimental realization of a 2X-filter and a 2X-half-filter, improvements of the experimental procedures by extension of the phase cycling schemes, and observation of small heteronuclear long-range spin–spin couplings in macromolecules. Practical applications are described for studies of the metal coordination in the protein metallothionein 2 from rat liver.

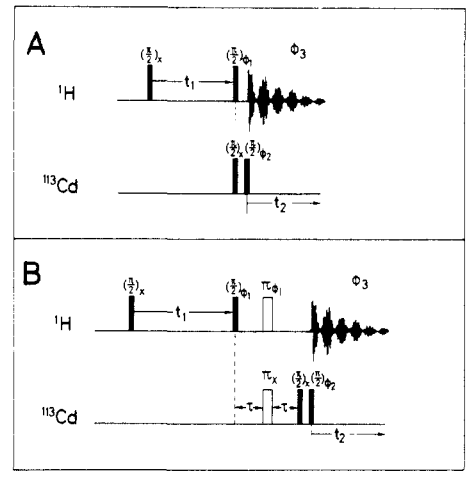

Figure 1. Experimental schemes for recording homonuclear ^1H COSY spectra with a heteronuclear filter (A) or half-filter (B). The delay between the two 90° X pulses is set to 3 μs to allow for the switching of the phase. τ is equal to $^1/_4J_{HX}$. These schemes can be used with different phase cycles for ϕ_2 to produce X-filters or 2X-filters (see Tables II and III and text).

Table II. Phase Cycle for ^1H COSY with an X-Filter (Figure 1A) or an X(ω_2)-Half-Filter (Figure 1B)

Φ_1	Φ_2	Φ_3	Φ_1	Φ_2	Φ_3
x	x	+	$-x$	x	+
x	$-x$	−	$-x$	$-x$	−
x	$-x$	−	$-x$	$-x$	−
x	x	+	$-x$	x	+

Metallothioneins represent a class of low molecular weight proteins consisting of a polypeptide chain with ca. 60 amino acid residues. They are uniquely designed to bind metal ions by complexation with cysteinyl residues and are believed to play important physiological roles in the detoxification of mammalian organisms upon intoxication with, e.g., Cd^{2+} or Hg^{2+}.[9] The recent determination of the three-dimensional structures of two homologous metallothioneins in single crystals[10] and in solution[11,12] resulted in an intriguing situation. While both structures contain two clusters with three and four metal ions, respectively, the topology of the metal coordination by the polypeptide chain is different in the crystal structure and in solution. In this context heteronuclear filter techniques are of interest, since they can yield direct information on the metal binding to cysteinyl residues in these proteins. Working with [$^{113}Cd_7$]-MT2 from rat liver (^{113}Cd has a nuclear spin $I = {}^1/_2$), which was previously characterized in detail by more conventional NMR experiments[13,14] and is therefore a suitable test molecule, we demonstrate the use of

(2) (a) Dubs, A.; Wagner, G.; Wüthrich, K. *Biochim. Biophys. Acta* **1979**, *577*, 177–194. (b) Nagayama, K.; Wüthrich, K. *Eur. J. Biochem.* **1981**, *114*, 365–374. (c) Wagner, G.; Anil Kumar; Wüthrich, K. *Eur. J. Biochem.* **1981**, *114*, 375–384. (d) Wüthrich, K.; Wider, G.; Wagner, G.; Braun, W. *J. Mol. Biol.* **1982**, *155*, 311–319. (e) Billeter, M.; Braun, W.; Wüthrich, K. *J. Mol. Biol.* **1982**, *155*, 321–346. (f) Wagner, G.; Wüthrich, K. *J. Mol. Biol.* **1982**, *155*, 347–366. (g) Wider, G.; Lee, K. H.; Wüthrich, K. *J. Mol. Biol.* **1982**, *155*, 367–388.

(3) (a) Hare, D. R.; Wemmer, D. E.; Chou, S. H.; Drobny, G.; Reid, B. R. *J. Mol. Biol.* **1983**, *171*, 319–336. (b) Scheek, R. M.; Russo, N.; Boelens, R.; Kaptein, R.; van Boom, H. *J. Am. Chem. Soc.* **1983**, *105*, 2914–2916. (c) Feigon, J.; Leupin, W.; Denny, W. A.; Kearns, D. R. *Biochemistry* **1983**, *22*, 5943–5951.

(4) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

(5) (a) Braun, W.; Wider, G.; Lee, K. H.; Wüthrich, K. *J. Mol. Biol.* **1983**, *169*, 921–948. (b) Williamson, M. P.; Havel, T. F.; Wüthrich, K. *J. Mol. Biol.* **1985**, *182*, 295–315. (c) Kline, A. D.; Braun, W.; Wüthrich, K. *J. Mol. Biol.* **1986**, *189*, 377–382. (d) Zuiderweg, E. R. P.; Billeter, M.; Kaptein, R.; Boelens, R.; Scheek, R. M.; Wüthrich, K. *Progress in Bioorganic Chemistry and Molecular Biology*; Ovchinnikov, Yu. A., Ed.; Elsevier: New York, 1984; pp 65–70.

(6) (a) Markley, J.; Putter, I.; Jardetzky, O. *Science (Washington, D.C.)* **1968**, *161*, 1249–1251. (b) Richarz, R.; Tschesche, H.; Wüthrich, K. *Biochemistry* **1980**, *19*, 5711–5715. (c) LeMaster, D. M.; Richards, F. M. *Biochemistry* **1985**, *24*, 7263–7269. (d) Griffey, R. H.; Redfield, A. G.; Loomis, R. E.; Dahlquist, F. W. *Biochemistry* **1985**, *24*, 817–822. (e) Live, D. H.; Davis, D. G.; Agosta, W. C.; Cowburn, D. *J. Am. Chem. Soc.* **1984**, *106*, 6104–6105. (f) Weiss, M. A.; Redfield, A. G.; Griffey, R. H. *Proc. Natl. Acad. U.S.A.* **1986**, *83*, 1325–1329.

(7) Wörgötter, E.; Wagner, G.; Wüthrich, K. *J. Am. Chem. Soc.* **1986**, *108*, 6162–6167.

(8) (a) Otting, G.; Senn, H.; Wagner, G.; Wüthrich, K. *J. Magn. Reson.* **1986**, *70*, 500–505. (b) Senn, H.; Otting, G.; Wüthrich, K. *J. Am. Chem. Soc.* **1987**, *109*, 1090–1092.

(9) Kägi, J. H. R.; Nordberg, M. *Metallothionein*; Birkhäuser: Basel, Switzerland, 1979.

(10) Furey, W. F.; Robbins, A. H.; Clancy, L. L.; Winge, D. R.; Wang, B. C.; Stout, C. D. *Science (Washington, D.C.)* **1986**, *231*, 704–710.

(11) Frey, M. H.; Wagner, G.; Vašák, M.; Sørensen, O. W.; Neuhaus, D.; Wörgötter, E.; Kägi, J. H. R.; Ernst, R. R.; Wüthrich, K. *J. Am. Chem. Soc.* **1985**, *107*, 6847–6851.

(12) (a) Neuhaus, D.; Wagner, G.; Vašák, M.; Kägi, J. H. R.; Wüthrich, K. *Eur. J. Biochem.* **1985**, *151*, 257–273. (b) Wagner, G.; Neuhaus, D.; Wörgötter, E.; Vašák, M.; Kägi, J. H. R.; Wüthrich, K. *Eur. J. Biochem.* **1986**, *157*, 275–289. (c) Braun, W.; Wagner, G.; Wörgötter, E.; Vašák, M.; Kägi, J. H. R.; Wüthrich, K. *J. Mol. Biol.* **1986**, *187*, 125–129.

(13) Wörgötter, E.; Wagner, G.; Vašák, M.; Kägi, J. H. R.; Wüthrich, K. *Eur. J. Biochem.* **1987**, *167*, 457–466.

(14) Vašák, M.; Wörgötter, E.; Wagner, G.; Kägi, J. H. R.; Wüthrich, K. *J. Mol. Biol.* **1987**, *196*, 711–719.

heteronuclear filters for the direct, separate identification of cysteine ligands bound to a single $^{113}Cd^{2+}$ ion and of bridging cysteines bound to two $^{113}Cd^{2+}$ ions.

Heteronuclear Filters for 2D ^{1}H NMR

Table I lists six heteronuclear filters for editing of homonuclear 2D ^{1}H NMR spectra with respect to scalar couplings J_{HX}. The X-filter[7] selects for heteronuclear two-quantum coherence. The resulting homonuclear 2D ^{1}H NMR spectra, e.g., COSY, NOESY, or TOCSY, contain only diagonal peaks of protons with nonvanishing scalar coupling J_{HX} and cross peaks connecting two protons with nonvanishing coupling to the *same* X spin. X-Half-filters[8] select the same order of coherence but only in one time domain, i.e. t_1 or t_2, so that the resulting homonuclear 2D ^{1}H NMR spectrum contains also the cross peaks connecting the X-coupled protons with the protons without heteronuclear coupling. Parts A and B of Figure 1 show the basic experimental schemes for ^{1}H COSY with an X-filter and an X(ω_2)-half-filter, respectively. The two experiments include the same sequence of radio-frequency pulses, but the X(ω_2)-half-filter contains an additional delay of duration 2τ between the mixing pulse of the homonuclear ^{1}H COSY experiment and the heteronuclear filter, with a refocusing 180° pulse on both ^{1}H and X. Corresponding schemes for NOESY and TOCSY, as well as for the X(ω_1)-half-filter, have been presented elsewhere.[7,8]

In previous applications of X-filters for studies of small molecules, we used the four-step phase cycle of the upper four rows in Table II combined with CYCLOPS[15] and time-proportional phase incrementation (TPPI) of the first pulse.[16] The three alternating phases ϕ_1–ϕ_3 govern clearly defined functions. The phase cycle for ϕ_1 is that of the corresponding 2D ^{1}H NMR experiment (the 180° (^{1}H) refocusing pulse in the middle of the additional delay of the half-filter experiment is cycled along with ϕ_1). ϕ_2 is the phase cycle for the second 90° pulse on the X spins, which determines the type of the resulting heteronuclear filter. ϕ_3, finally, is the receiver cycling consisting of alternating addition and subtraction of the free induction decays. Applications of the heteronuclear filters with proteins require the observation of very weak signals, which turned out to be difficult because the aforementioned four-step phase cycle often gave incomplete suppression of unwanted peaks. Inspection of Figure 1 provides the explanation: The X-filter in Figure 1A consists of a pair of successive 90° pulses on the X spins. For $\phi_2 = x$ this pair of pulses inverts heteronuclear antiphase coherence, e.g. I_xS_z,[17] and for $\phi_2 = -x$, they leave it unchanged. If the free induction decays obtained with $\phi_2 = x$ and $\phi_2 = -x$ are subtracted by the phase cycle for ϕ_3 (Table II), the experiment selects for heteronuclear antiphase coherence that contains an uneven number of S_z factors in the product operator representation of the density operator (e.g., I_xS_z or $I_xS_{1z}S_{2z}S_{3z}$, whereby only the first term needs to be considered in practice). This eliminates ^{1}H coherence from protons not coupled to X (e.g., $I_{1x}I_{2z}$). Difficulties arise since, to achieve a workable sensitivity, the relaxation delay in NMR experiments with biological macromolecules is usually chosen too short to allow for complete T_1 relaxation between subsequent scans. In COSY the axial peak suppression scheme with the alternation of ϕ_2 from $+x$ to $-x$ (Table II) then affects the ^{1}H magnetization at the start of successive scans; e.g., the first scan of the phase cycle (Table II) starts from a different z-magnetization than the second scan, and the third scan starts from a different z-magnetization than the fourth scan. Since the X-filter relies on subtractive combination of successive scans, the elimination of coherence from ^{1}H spins that are not coupled to an X spin will thus be incomplete, leading to the appearance of spurious signals. This can be rectified if a second cycle of scans is coadded with permutation of the phase cycle for the receiver (ϕ_3) (Table II). The error arising from incomplete ^{1}H relaxation, which is accumulated in the first four scans, is thus corrected, since during the second cycle the same error is accumulated with opposite sign. (Since the receiver phase is directly correlated with the pulses on X, ϕ_2 must also be permuted together with ϕ_3; see Table II.)

The extended phase cycle of Table II illustrated here for COSY should be used with all 2D ^{1}H NMR experiments that involve variation of the phases for the ^{1}H pulses between different recordings with the same t_1 value. Storage of scans with positive and negative sign for ϕ_3 in two different memory locations enables the generatior with a single experimental setup, of the X-filtered spectrum and the corresponding normal 2D ^{1}H NMR spectrum (for details see ref 7).

The second group of three filters in Table I selects for heteronuclear three-quantum coherence of one ^{1}H and two X spins. For the 2X-filter the resulting 2D ^{1}H NMR spectrum contains only diagonal peaks of protons with nonvanishing scalar couplings to two X spins and cross peaks connecting two protons, both of which have nonvanishing couplings, to the *same* two X-spins. 2X-Half-filters select only in either ω_1 or ω_2. Therefore, the cross peaks connecting the protons coupled to two X-spins with the protons having either one or no heteronuclear coupling are also present in the resulting 2D ^{1}H NMR spectrum. 2X-Filters use the same basic experimental schemes as the X-filters, with the phase cycling given in Table III. The basic phase cycle consists of four steps for ϕ_2 (i.e. x, y, $-x$, and $-y$) as compared to two steps for the X-filter (Table II). Correspondingly, a fourfold permutation of the phase cycle is needed to suppress artefacts from incomplete ^{1}H relaxation between scans (Table III).

Table III. Phase Cycle for ^{1}H COSY with a 2X-Filter (Figure 1A) or a 2X(ω_2)-Half-Filter (Figure 1B)

Φ_1	Φ_2	Φ_3	Φ_1	Φ_2	Φ_3
x	x	+	$-x$	x	+
x	y	−	$-x$	y	−
x	$-x$	+	$-x$	$-x$	+
x	$-y$	−	$-x$	$-y$	−
x	y	−	$-x$	y	−
x	$-x$	+	$-x$	$-x$	+
x	$-y$	−	$-x$	$-y$	−
x	x	+	$-x$	x	+
x	$-x$	+	$-x$	$-x$	+
x	$-y$	−	$-x$	$-y$	−
x	x	+	$-x$	x	+
x	y	−	$-x$	y	−
x	$-y$	−	$-x$	$-y$	−
x	x	+	$-x$	x	+
x	y	−	$-x$	y	−
x	$-x$	+	$-x$	$-x$	+

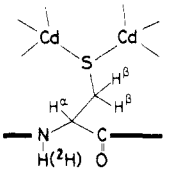

Figure 2. Schematic drawing of a bridging cysteine in MT2, which is bound to two metal ions via the sulfur. The thick horizontal line indicates the polypeptide chain. For the experiments with nonlabile hydrogen atoms, the amide protons were exchanged against deuterium.

Results

This section describes applications of heteronuclear filters (Table I) for identification of the ^{1}H spin systems of the metal-bound amino acid residues in rat liver [$^{113}Cd_7$]–MT2.[10,14] This provided a controlled test with a complex macromolecular system for these filtering techniques, since sequence-specific resonance assignments for the polypeptide chain and identification of the amino acid residues bound to ^{113}Cd were previously obtained with homonuclear 2D ^{1}H NMR[13] and with heteronuclear [^{113}Cd,^{1}H]-COSY

(15) Hoult, D. I.; Richards, R. E. *Proc. R. Soc. London, A* **1975**, *344*, 311–320.
(16) Marion, D.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967–974.
(17) $X = S$ is used in the product operator terms. Sørensen, O. W.; Eich, G. W.; Levitt, M. H.; Bodenhausen, G.; Ernst, R. R. *Prog. Nucl. Magn. Reson. Spectrosc.* **1983**, *16*, 163–192.

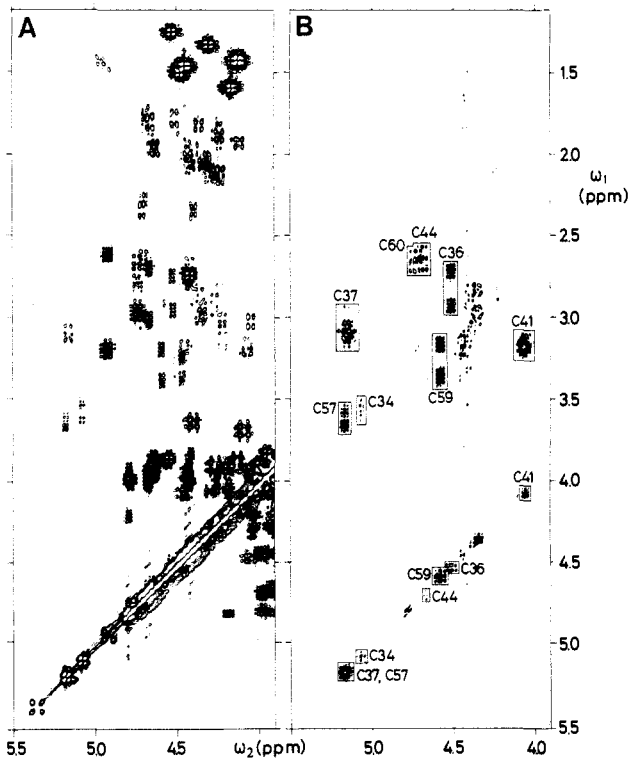

Figure 3. Region (ω_1 = 1.1–5.5 ppm, ω_2 = 3.9–5.5 ppm) of phase-sensitive 360-MHz 2D ^1H NMR spectra of a 10 mM solution of rat liver [$^{113}Cd_7$]–MT2 in ^2H_2O (p^2H 7.0, 25 °C, internal reference TSP). Both positive and negative levels are shown: (A) conventional ^1H COSY, (B) ^1H COSY recorded with an X-filter (X = ^{113}Cd). The resonances originating from ^{113}Cd-bound cysteines are framed and identified with the sequence positions. The diagonal peak at 4.75 ppm corresponds to the incompletely suppressed solvent resonance; the diagonal and cross peaks between ω_2 = 4.35 and 4.45 ppm correspond to additional Cys signals, which are not individually resolved.

and [^{113}Cd,^1H,^1H]-RELAYED-COSY.[14] All metal ions were found to be bound exclusively to cysteinyl side chains. The chemical structure of a *bridging* cysteine in MT2 is shown in Figure 2. The two ^{113}Cd spins bound to the sulfur are related by a three-bond coupling with the β-protons of Cys, $^3J_{^{113}CdH^\beta}$, and by a four-bond coupling with the α-proton, $^4J_{^{113}CdH^\alpha}$. The nonbridging cysteines, which are bound to a single $^{113}Cd^{2+}$ ion, give rise to similar three-bond and four-bond couplings.

Figure 3A shows that part of a phase-sensitive ^1H COSY spectrum of rat MT2 that contains the cross peaks between the α-protons (along ω_2) and the β-protons (along ω_1) and the diagonal peaks of the α-protons.[13] Figure 3B shows the same spectral region of a ^1H COSY spectrum recorded with a ^{113}Cd-filter. Only diagonal peaks of and cross peaks between protons coupled to ^{113}Cd can be seen, which leads to a notable simplification of the spectrum. Each amino acid ^1H spin system represented by cross peaks or diagonal peaks in the spectrum of Figure 3B can thus be attributed to a metal-bound amino acid residue. In a different region of the ^{113}Cd-filtered ^1H COSY spectrum, the H^β–$H^{\beta'}$ cross peaks of Cys spin systems could also be observed. However, the observation of H^α–H^β cross peaks and H^α diagonal peaks in the region shown in Figure 3B is of special interest, since according the selection rules (Table I) the appearance of these peaks requires that both the β-proton and the α-proton of Cys (Figure 2) have resolved spin–spin couplings with ^{113}Cd.

Two experiments enabling direct identification of bridging cysteines in MT2 are shown in Figure 4. The region from a ^1H COSY spectrum recorded with a 2X-filter (X = ^{113}Cd) (Figure 4A) contains a peak near the diagonal at ca. 3.6 ppm and a well-resolved cross peak. Diagonal peaks and cross peaks observed with a 2X-filter correspond to the presence of product operator terms $I_xS_{1z}S_{2z}$ and $I_{1x}I_{2z}S_{1z}S_{2z}$ before the mixing pulse, respectively (Table I), which are selected by the experiment of Figure 1A with

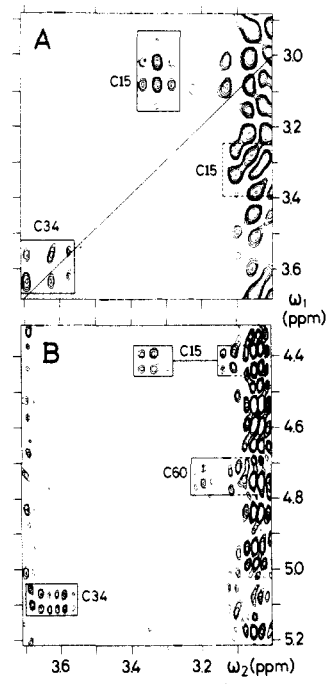

Figure 4. (A) Contour plot of the H^β–$H^{\beta'}$ cross-peak region for AMX spin systems[4] from a ^1H COSY spectrum of rat liver [$^{113}Cd_7$]–MT2 recorded with a 2X-filter (X = ^{113}Cd). The position of the diagonal is indicated with the solid line. (B) Part of the H^α–H^β cross-peak region for AMX spin systems in a ^1H COSY spectrum recorded with a 2X-(ω_2)-half-filter (X = ^{113}Cd). In both spectra the identified diagonal peaks and cross peaks are framed and labeled with the amino acid one-letter symbol and the sequence position. Broken lines indicate the positions of cross peaks that are buried in the t_1 noise bands of the lysine ϵ-protons at 3.05 ppm.

the phase cycle of Table III. Their presence in Figure 4A shows that they are part of a ^1H spin system of an amino acid residue bound to two ^{113}Cd spins. Note that, in spite of the fact that the H^β–$H^{\beta'}$ cross peak of Cys-15 with the low-field H^β along ω_2 is prominently seen, the diagonal peak of the low-field β-proton is clearly absent. This is a consequence of the facts that the coupling of this proton with ^{113}Cd is smaller than for the high-field β-proton and, as usual in 2D ^1H NMR,[4] the digital resolution along ω_2 is higher than along ω_1. The peak attributed to Cys-34 corresponds to a superposition of the diagonal and cross peaks for β-CH_2 in this residue, so that the relative intensities of the individual components are difficult to assess. Figure 4B shows the spectral region containing H^α–H^β cross peaks of AMX spin systems from a ^1H COSY spectrum recorded with a 2X(ω_2)-half-filter (X = ^{113}Cd). The cross peaks from three AMX spin systems can be observed, whereas in the spectrum of Figure 4A this region was empty. Since at least one of the correlated protons must have nonvanishing couplings to two ^{113}Cd spins (Table I), the experiment of Figure 4B is an alternative to that of Figure 4A for a direct identification of bridging cysteines in MT2. It confirms the presence of the bridging cysteines 15 and 34 and further identifies Cys-60 as a bridging cysteine.

Discussion

From earlier studies[10,13,14] it is known that the 7 ^{113}Cd ions in rat MT2 are bound by 12 singly coordinated cysteines and 8 bridging cysteines. Since the ^1H chemical shifts for all 20 cysteines are known,[13] the locations of all diagonal peaks and cross peaks expected in the spectra of part B of Figure 3 and parts A and B of Figure 4 can be accurately predicted. In the ^{113}Cd-filtered ^1H COSY spectrum, peaks may originate from any of the 20 metal-bound cysteines. In Figure 3B, eight of these are represented by well-resolved cross peaks and diagonal peaks, and at least three additional residues contribute to the mutually overlapped peaks along $\omega_2 \approx 4.35$ ppm. The remaining cysteines are not seen in this spectral region, probably because the couplings $^4J_{^{113}CdH^\alpha}$ are not resolved. A more complete set of cysteine peaks was seen in

the H^{β}–$H^{\beta'}$ cross-peak region of the same spectrum (not shown), which was, however, only partially resolved because of the small dispersion of the Cys β-CH_2 chemical shifts in rat MT2.[13] Of the eight bridging cysteines, two are observed in the 2X-filtered ^1H COSY spectrum (Figure 4A) and three with the 2X(ω_2)-half-filter (Figure 4B). The locations predicted for the remaining five bridging cysteines were screened in both of these spectra, and it was found that these positions were masked by t_1 noise or other spurious signals.

The principal interest in these results is that the NMR lines of protons coupled to an NMR-active isotope can be identified without comparison with the spectrum obtained by labeling with a NMR-inactive isotope of the same element. For example, in metallothionein the metal ligands were so far identified by comparison of the ^1H NMR spectra of two protein preparations reconstituted, respectively, with ^{113}Cd ($I = {}^1/_2$) and ^{112}Cd ($I = 0$).[18] With the availability of heteronuclear filters, only [$^{113}Cd_7$]–MT2 would have to be prepared. In the present experiments of Figures 3 and 4, only part of the 20 metal-bound cysteines were observed. For a proper assessment of the potentialities of these techniques, it must be considered, however, that a rather old 360-MHz NMR instrument was used for these studies. With a modern spectrometer at 500 or 600 MHz, the spectral resolution in the H^{β}–$H^{\beta'}$ region of X-filtered ^1H COSY spectra and the sensitivity for observation of the peaks in 2X-filtered and 2X(ω_2)-half-filtered ^1H COSY would be greatly improved, so that a complete set of resonances might be obtained.

The 2X-filter can yield a direct identification of bridging cysteines, since each cross peak in ^1H COSY recorded with a 2X-filter may have nonzero intensity only if all five active couplings are resolved, and diagonal peaks with a 2X-filter or cross peaks in ^1H COSY recorded with a 2X(ω_2)-half-filter require three active couplings (Table I). Because of the more stringent selection rules, one might, as a general rule, expect that bridging cysteines would escape detection more easily in the cross-peak regions of 2X-filter ^1H COSY than either on the diagonal of 2X-filter ^1H COSY or in 2X(ω_2)-half-filter ^1H COSY. In the present experiments this receives support from the fact that the bridging Cys-60 was only seen in Figure 4B. In contrast, the previously discussed absence of the diagonal peak of Cys-15 in Figure 4A illustrates that the selection of the experimental parameters may favor observation of peaks with more stringent selection rules over others. Also, because of the additional delay time in the half-filter experiment (Figure 1), corresponding peaks may have higher intensity when observed with a 2X-filter than with a 2X(ω_2)-half-filter.

The following are some general comments on the practice of heteronuclear filtering. First, applications are of course not limited to systems containing ^{113}Cd. For example, the use of heteronuclear filters in ^1H NOESY experiments with biomacromolecules interacting with ^{13}C- or ^{15}N-labeled ligands offers most exciting prospects for future studies. Second, it is clear that heteronuclear filters are a complementation and not a substitute for heteronuclear correlation experiments, since in contrast to the latter they cannot characterize the X-spins by their chemical shifts. Third, quite generally, the diagonal peaks in ^1H spectra recorded with heteronuclear filters may contain valuable information (Table I). In particular, they enable the observation of heteronuclear couplings also with ^1H singlets or with ^1H multiplets having very small homonuclear coupling constants. Fourth, the extended permutation phase cycles introduced in this paper (Tables II and III) should always be used when the relaxation delay between individual scans is too short to allow for complete T_1 relaxation. This ensures that the signals due to spins without coupling to X are completely suppressed and that clean multiplet fine structures[7] are obtained. The increased length of the phase cycle is not usually a limiting factor in NMR studies of biological macromolecules, since the minimum number of scans required by the phase cycle rarely exceeds that needed to achieve a workable signal-to-noise ratio.

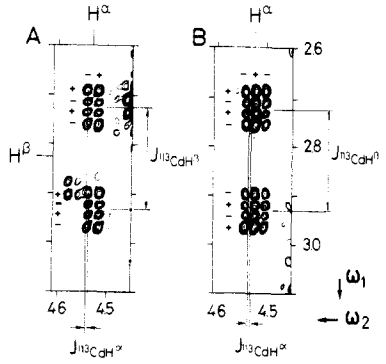

Figure 5. Fine structure from heteronuclear couplings $J_{^{113}Cd^1H}$ and homonuclear couplings $J_{^1H^1H}$ of one of the two H^{α}–H^{β} cross peaks of Cys-36 in rat liver [$^{113}Cd_7$]–MT2: (A) conventional ^1H COSY, (B) ^1H COSY with ^{113}Cd-filter. The two heteronuclear couplings $^3J_{^{113}CdH^{\beta}}$ and $^4J_{^{113}CdH^{\alpha}}$ are indicated with arrows, and the chemical shifts for H^{α} and H^{β} are also identified. + and – signs are drawn for each row of resolved fine structure peaks along ω_1 and for each column of resolved peaks along ω_2. The sign of each individual multiplet component can be calculated as the product of the signs for the respective row and column.

In metallothioneins the appearance of H^{α}–H^{β} cross peaks in X-filtered ^1H COSY is of practical interest for studies of the cysteine spin systems, since these peaks are dispersed over a wider chemical shift range than the H^{β}–$H^{\beta'}$ cross peaks. Similar practical advantages may be expected also for other systems with small long-range heteronuclear couplings. Besides the practical aspects, the H^{α}–H^{β} cross peaks are of fundamental interest to the spectroscopist, since they present a clear manifestation of small couplings that have not been detected by visual inspection of regular ^1H COSY spectra of MT2. (We also recorded experiments in H_2O solution of rat [$^{113}Cd_7$]–MT2 to search for cross peaks manifesting ^{113}Cd–amide proton five-bond couplings (Figure 2). No such peaks could so far be detected with the available equipment.) This can be well appreciated on the basis of earlier systematic studies on the manifestations of homonuclear and heteronuclear couplings in the cross-peak multiplets of ^1H COSY spectra recorded without[18] or with[7] heteronuclear filtering. Formally the normal ^1H COSY cross peaks for X-coupled cysteine protons in [$^{113}Cd_7$]–MT2 correspond to those observed for homonuclear spin systems in E.COSY,[19] where only directly connected transitions give rise to multiplet components. This is due to the fact that the ^{113}Cd spins are not influenced by the ^1H mixing pulse. Individual fine-structure components may thus be differently displaced along ω_1 or ω_2 by the heteronuclear couplings, which leads to characteristically asymmetric ("incomplete") multiplets.[4,7,18] If the heteronuclear coupling is small compared to the natural line width, these shifts may be practically invisible in normal ^1H COSY. Such a situation is shown in Figure 5A for a H^{α}–H^{β} cross peak of Cys-36. This cross peak is well separated in a normal ^1H COSY spectrum, and the β-proton chemical shifts are sufficiently different to allow a first-order analysis. The large coupling $^3J_{^{113}CdH^{\beta}} = 76$ Hz produces a pronounced separation into two components along ω_1. In contrast, the right-hand shift of the lower component relative to the upper one, which corresponds to the $^4J_{^{113}CdH^{\alpha}}$ value of 1.8 Hz, can hardly be recognized in Figure 5A. In ^1H experiments recorded with an X-filter all heteronuclear couplings are active, producing cross-peak patterns with a complete set of multiplet components corresponding to ^1H transitions with both X-spin polarizations during both t_1 and t_2.[7] In the resulting H^{α}–H^{β} cross-peak fine structure (Figure 5B), two positive components along ω_2 nearly coincide in the cross-peak center, giving rise to intense peaks even though the ^{113}Cd–H^{α} couplings are rather small. In contrast to normal ^1H COSY (Figure 5A), a detailed analysis of the multiplet structure is not required, since the observation of the cross peak in Figure 5B constitutes in itself unambiguous evidence for the presence of a nonvanishing hetero-

(18) Neuhaus, D.; Wagner, G.; Vašák, M.; Kägi, J. H. R.; Wüthrich, K. *Eur. J. Biochem.* **1984**, *143*, 659–667.

(19) Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1985**, *107*, 6394–6396.

nuclear $^4J_{^{113}CdH^\alpha}$ coupling (Table I).

Experimental Section

Rat liver [$^{113}Cd_7$]-MT2 was prepared as described previously.[14] For the NMR measurements, ca. 0.01 M solutions of the protein in ^2H_2O containing 0.02 M [$^2H_{11}$]Tris/^{2}HCl at p^{2}H 7.0 and 0.05 M KCl were used. The labile protons had been exchanged with deuterium by twice freeze-drying and redissolving the material in ^2H_2O. The sample was kept under argon.

For the ^{1}H COSY spectrum with ^{113}Cd-filter, 224 scans were collected per t_1 value, with a relaxation delay of 1.6 s, including the acquisition time. The complete eight-step phase cycle of Table II was combined with CYCLOPS,[15] so that the total phase-cycling scheme consisted of 32 steps. For both spectra in Figure 3, the digital resolution before zero-filling was 3.2 Hz along ω_2 and 5.1 Hz along ω_1.

For the ^{1}H COSY spectra with a 2X-filter or a 2X(ω_2)-half-filter, 384 scans were acquired per t_1 value. The relaxation delay was 1.8 s, including the acquisition time. For the 2X(ω_2)-half-filter, a tuned delay of τ = 20 ms (Figure 1B) was used, which corresponds to a J_{HX} value of 12.5 Hz. A 128-step phase cycle was used for both experiments, corresponding to the combination of the complete 32-step phase cycling scheme of Table III with CYCLOPS.[15] Before zero-filling, the digital resolution was 4.3 Hz along ω_2 and 8.6 Hz along ω_1.

All experiments were recorded with time-proportional phase incrementation (TPPI) of the first pulse[16] on a Bruker AM 360 spectrometer equipped with an ASPECT 3000 computer and modified to allow ^{1}H observation and ^{113}Cd pulsing. The temperature was 25 °C. For all the spectra displayed in Figures 3–5, fourfold zero-filling was applied in both dimensions. Prior to the phase-sensitive Fourier transformation, the time domain data were multipled in both dimensions with sine-bell windows.[20]

Acknowledgment. We thank M. Sutter for the preparation of the biological materials and E. Huber and E.-H. Hunziker-Kwik for the careful preparation of the manuscript and the illustrations. Financial support by the Schweizerischer Nationalfonds (Projects 3.198.0.85 and 3.164.0.85) and EMBO (fellowship to E.W.) is gratefully acknowledged.

Registry No. Cys, 52-90-4; Cd, 7440-43-9.

(20) DeMarco, A.; Wüthrich, K. *J. Magn. Reson.* 1976, *24*, 201–204.

Chemical Reactions of Imidazole with Metallic Silver Studied by the Use of SERS and XPS Techniques

Gi Xue,*† Quinpin Dai,‡ and Shuangen Jiang‡

Contribution from the Department of Chemistry and The Analysis Center, Nanjing University, Nanjing, The People's Republic of China. *Received August 17, 1987*

Abstract: Chemical reactions of imidazole with metallic silver have been studied by surface-enhanced Raman scattering (SERS), infrared (IR), and X-ray photoelectron spectroscopy (XPS) techniques. We found that imidazole can react with metallic silver directly in the presence of oxygen. The product formed is (imidazolato)silver(I), which covers the surface of metal as a monolayer. The reaction scheme and the structural features of the product are discussed.

Imidazole (ImH) is of considerable interest as a ligand in that its presence in many biological systems provides a potential binding site for metal ions. A large amount of work has been published on imidazole and related ligands and the complexing properties of such compounds, both in the solid state and in solution.[1] The unprotonated imidazole usually functions as a ligand in solutions near neutrality via the unshared pair of electrons on nitrogen. Complexes including Ag(ImH)Cl, $Cu(ImH)_4Cl_2$, $Co(ImH)_2Cl_2$, $Ni(ImH)_2Cl_2$, etc., have been studied extensively.[2-6] In sufficiently basic media the conjugate base of imidazole, Im^-, is formed and may function as a ligand too. The tendency then is for formation of an "inner" complex of stoichiometry $M^{2+}(Im^-)_2$ or $M^+(Im^-)$ with dipositive metal ions. These materials are usually insoluble and generally are considered to be polymeric in nature. Examples reported include bis(imidazolato)copper(II) and (imidazolato)silver (I).[7-11]

The complexes of Im^- are usually prepared from the metal ion and imidazole in basic solution.[11] In this work we report our finding about the formation of (imidazolato)silver(I) by direct reaction of metallic silver and ImH.

Imidazole and its derivatives are also of interest as corrosion inhibitors for metals and alloys. The molecular structure and anticorrosion mechanism have been studied by many authors but are still not well understood.[12-15] The consensus is that imidazole can react with surface oxides on metal to form a metal–azole coordination film.[16-19] Relatively few reports exist in the literature concerning the direct reaction of imidazole with metallic copper or silver.

The observation on enormously enhanced cross sections (factors up to 10^6) for Raman scattering from molecules adsorbed on metal surface is one of the most important discoveries in the field of surface science in the last few years.[20-22] We have taken advantage of this sensitivity of SERS to study the molecular structure of the imidazole residue on silver colloids, resulting in the discovery

†Department of Chemistry.
‡The Analysis Center.

(1) Sundberg, R. J.; Martin, R. B. *Chem. Rev.* 1974, *74*, 471.
(2) Eilbeck, W. J.; Holmes, F.; Taylor, C. E.; Underhill, A. E. *J. Chem. Soc. A* 1968, 128.
(3) Eilbeck, W. J.; Holmes, F.; Underhill, A. E. *J. Chem. Soc. A.* 1967, 757.
(4) Holmes, F.; Jones, K. M.; Torrible, E. G. *J. Chem. Soc. A* 1961, 4790.
(5) Brooks, P.; Davidson, N. *J. Am. Chem. Soc.* 1960, *82*, 2118.
(6) Tanford, C.; Wagner, M. L. *J. Am. Chem. Soc.* 1953, *75*, 434.
(7) Jarvis, J. A. J.; Wells, A. F. *Acta Crystallogr.* 1960, *13*, 1027.
(8) Brown, G. P.; Aftergut, S. *J. Polym. Sci., Part A* 1964, *2*, 1839.
(9) Bauman, J. E., Jr.; Wang, J. C. *Inorg. Chem.* 1964, *3*, 368.
(10) Sigwart, P. K.; Hemmerich, P. *Helv. Chim. Acta* 1970, *53*, 177.
(11) Reference 8.
(12) Wall, K. H.; Davies, I. *J. Appl. Chem.* 1965, *15*, 389.
(13) Tompkins, H. G.; Sharma, S. P. *SIA, Surf. Interface Anal.* 1982, *4*, 61.
(14) Yoshida, S.; Ishida, H. *J. Adhes.* 1984, *16*, 217.
(15) Yoshida, S.; Ishida, H. *Appl. Surf. Sci.* 1985, *20*, 497.
(16) Cotton, J. B.; Scholes, I. R. *Br. Corros. J.* 1976, *2*, 1.
(17) Yoshida, S.; Ishida, H. *J. Mater. Sci.* 1984, *19*, 2323.
(18) McCrory-Joy, C.; Rosamilia, J. M. *J. Electroanal. Chem. Interfacial Electrochem.* 1982, *136*, 108.
(19) Ito, M.; Takahushi, M. *Surf. Sci.* 1985, *158*, 609.
(20) Schatz, G. C.; Van Duyne, R. P. *Surf. Sci.* 1980, *101*, 425.
(21) Thierry, D.; Leygraff, C. *J. Electrochem. Soc.* 1985, *132*, 1009.
(22) Jeanmaire, D. L.; Van Duyne, R. P. *J. Electroanal. Chem. Interfacial Electrochem.* 1977, *84*, 1.

0002-7863/88/1510-2393$01.50/0