Surreptitious involvement of a metallacycle substituent in metal-mediated alkyne cleavage chemistry

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mass spectrum reflect the charge states of the protein in solution, then the spectrum will yield information regarding the conformational state of the protein.

The mass spectra of bovine cytochrome *c*, shown in Figure 1, were obtained[7] by electrospraying aqueous solutions over a range of pH values (2.6–5.2) where the cytochromes *c* are known to undergo conformational changes.[9-15] The mass spectrum obtained from a solution at pH 2.6 (Figure 1a) exhibits eight peaks, each one corresponding to a different protonation state of cytochrome *c*. These protonation states range from 11+ to 18+ with 16+ being the most intense. Mass spectra of this type, exhibiting ions with a wide distribution of charge states and a single maximum, are typical of the electrospray ionization mass spectra of proteins that have been reported.[5,6,8] Surprisingly, as the pH of the sprayed solution was increased to 3.0, a second maximum with a protonation state of 8+ appears in the mass spectrum (Figure 1b). The width of this second distribution is narrow and is largely composed of the 8+ ion peak. Upon further increase of the pH to 5.2, the intensity of the distribution centered around 16+ decreases substantially and a third distribution centering around 10+ (with protonation states ranging from 8+ to 12+) is observed to dominate the spectrum (Figure 1c). Determination of molecular mass from the observed mass-to-charge ratios confirmed that all the peaks designated as 8+ to 18+ arise from intact bovine cytochrome *c*. We interpret the dramatic changes observed in the cytochrome *c* mass spectra (Figure 1) to result from differences in the conformational states of the protein in solution.

At low pH, the protein unfolds (state A) so that it can accept a large number of protons (Figure 1a). As the pH is raised, some of the cytochrome *c* molecules fold into a relatively tight conformation (state B) that can accept far fewer protons and produces a second distribution centered at the 8+ charge state (Figure 1b). The simultaneous observation of two discrete distributions of ions with no ions having intermediate charge states provides evidence for a highly cooperative transition between the two conformations. The tight conformation, B, can readily be converted into the highly charged unfolded state, A, by the addition of a denaturing agent such as methanol. Upon a further increase in the pH, virtually all the protein molecules are converted into a second folded conformation (state C) that can accept a larger number of protons than B but a smaller number than A.

The acid unfolding of cytochrome *c* has been extensively studied by various other techniques including acid–base titrations,[9] optical rotation,[10] spectrophotometry,[9,11-15] circular dichroism,[15] fluorescence,[15] NMR,[16] temperature jump,[12,17] and viscometry.[14,15] The existence of at least three conformational states in acidic conditions has been reported.[9,11,12,15,17] The results of the present investigation also indicate the presence of three distinct conformational states of cytochrome *c* for electrosprayed solutions in the pH range 2.6–5.2. In the electrospray ionization process, small highly charged droplets are initially formed that rapidly evaporate before gas-phase ions are finally produced. Because the evolution of the effective pH of the rapidly evaporating charged droplets is not known, a direct correlation between the presently observed and previously reported conformational states cannot be made.

The technique has also been applied to the investigation of conformational changes in horse cytochrome *c*, bovine ubiquitin, and yeast ubiquitin induced by changes in pH and by addition of organic solvents.[18] Dramatic changes in the charge distributions were observed in each case that could be correlated with changes in protein conformation. It is noteworthy that the charge distributions of proteins containing disulfide bonds have also been observed to be increased by reduction of the disulfide bonds.[19]

Our findings demonstrate the viability of a new physical method for probing conformational changes in proteins. In addition, these studies provide the basis for a better understanding of the roles of solvent composition and protein conformation in the degree to which proteins are ionized in the electrospray process.

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(7) The aqueous protein solutions (without the addition of any buffers) were electrosprayed at room temperature through a 150-μm-i.d. stainless steel syringe needle, whose tip was etched to provide a sharp conical shape. The electrospray ionization mass spectrometer used in the present investigations has been described earlier.[8] All the experiments were performed under identical conditions, except for the amounts of acetic acid added to the spray solutions and the flow rates, which ranged between 0.15 μL/min at pH = 2.6 and 1.0 μL/min at pH = 5.2.

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Surreptitious Involvement of a Metallacycle Substituent in Metal-Mediated Alkyne Cleavage Chemistry

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Potential applications of metallacycles in organic synthesis[1] and, more recently, in the area of electronic materials[2] have stimulated extensive research into the properties and reactivity of this compound class. A virtually untapped source of metallacycle reactivity lies in the chemistry of the ring substituents, particularly the α-substituents due to their close proximity to adjacent coordination sites.[3,4] Herein we describe the iridiacycle-mediated alkyne cleavage reaction represented by eq 1. Although such a transformation is rare,[5] and remarkable in its own right, labeling studies have revealed a novel mechanism that includes surreptitious involvement of an α-metallacycle substituent.

$$M + H_2O + HC{\equiv}CR' \rightarrow MCO + H_3CR' \qquad (1)$$

When a wet chloroform-d_1 solution of $Ir(\overline{CR{=}CR\text{-}CR{=}CR})(PPh_3)_2(NCCH_3)_2^+BF_4^-$ (**1**)[6] (R = CO_2CH_3, 9.5 mM) and methyl propiolate (95 mM) is maintained at 23 °C for

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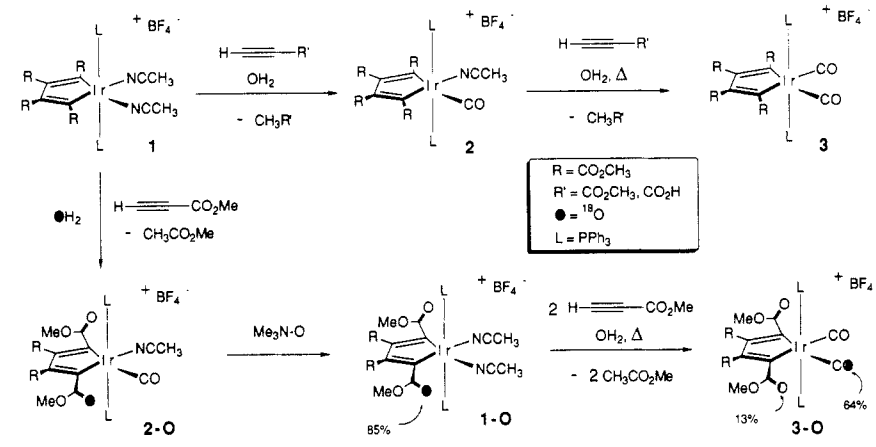
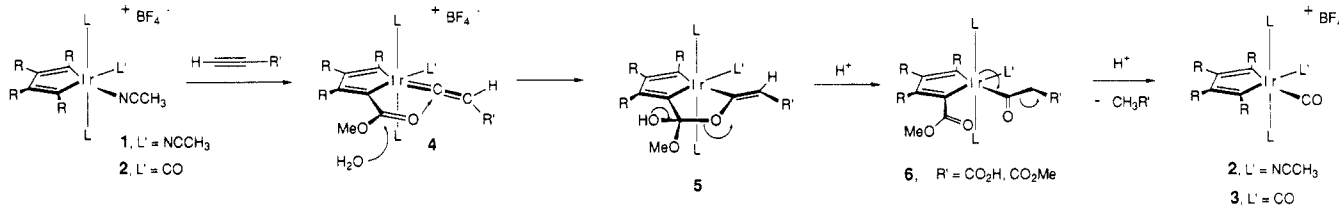
Scheme I



Scheme II



2 weeks, the carbonyl complex **2** (75%)[7] and methyl acetate (97%) are generated (Scheme I). When wet chloroform-d_1 solutions of **1** are heated at 74 °C in the presence of methyl propiolate, the bis(carbonyl) complex **3** (36%) and methyl acetate (158%, based on **1**) are formed. In the conversion to **1** to **3**, several unidentified iridium products are observed as well as the formation of methanol (120%, based on **1**). Complex **2** also generates **3** and methyl acetate from reaction with methyl propiolate at 74 °C. When propiolic acid is substituted for methyl propiolate, the reactions are much cleaner and the yields of iridium carbonyl complexes and acetic acid are in the 94–97% range.

The formation of the organic products in these reactions suggests that the source of the carbon monoxide ligand is the terminal alkyne carbon. In order to unambiguously determine the origin of the oxygen atom in the CO ligand, we ran the **1** → **2** conversion in the presence of $^{18}OH_2$. In order to avoid ^{18}O exchange into the alkyne substrate, we employed methyl propiolate rather than propiolic acid in the labeling studies. As anticipated, a FAB mass spectrum of the product, **2-O**, demonstrated 93% incorporation of oxygen-18 (m/e 1031; $M^+ - BF_4 - NCCH_3 + 2$). However, a fragment peak at m/e 1003 ($M^+ - BF_4 - NCCH_3 - CO + 2$) indicated that oxygen-18 enrichment (73 ± 10%) was not, as expected, at the carbon monoxide ligand. This was confirmed by the absence of an isotopic shift for ν(CO) in the IR spectrum of **2-O**. The location of the oxygen-18 enrichment was determined to be at one and only one of the methoxycarbonyl ring substituents by $^{13}C\{^1H\}$ NMR (125.7 MHz) spectroscopy on a 2:1 mixture of **2-O**:**2**. The *only* carbon resonance to exhibit an isotopic shift (4.5 Hz upfield) was a singlet at δ 169.0 (CO_2CH_3 of **2**).[8]

Treatment of **2-O** with $(CH_3)_3N^+-O^-$ in CH_3CN leads to the labeled bis(acetonitrile) complex **1-O**, with 85 ± 10% ^{18}O label at one of the ester substitutents. Upon reaction of **1-O** with methyl propiolate in wet chloroform at 74 °C, the bis(carbon monoxide) product **3-O** is generated. Mass spectral analysis of **3-O** indicates that 64% of the oxygen-18 label is located in the CO ligands (qualitatively confirmed by IR spectroscopy) and 13% remains at the methoxycarbonyl site.[9] The missing 8% of oxygen-18 label presumably washed out by OH_2 exchange into the labeled CO ligand.[10] The oxygen-18 label in the methoxycarbonyl group is therefore incorporated into the newly formed CO ligand in a highly selective manner, as initially suggested from the **1** to **2-O** conversion.

On the basis of the above product analyses and labeling results we propose the alkyne cleavage mechanism outlined in Scheme II. A reasonable first step is vinylidene ligand formation (**4**), a transformation of terminal alkynes with a great deal of precedent.[11-13] Interaction of the α-methoxycarbonyl oxygen with the electrophilic vinylidene carbon would result in activation toward nucleophilic attack by water to give **5**. The oxametallacycle ring in **5** then opens to give the β-oxoacyl complex **6**. In the conversion of **1** to **3**, the observed methanol and iridium side-product formation may be due to loss of methanol from **5** rather than ring opening. Fragmentation of the oxoacyl ligand in **6** generates the carbonyl ligand and organic product.[14] The 13 ± 10% label at the ester site of **3-O** presumably results from a minor amount of water attack directly at the vinylidene ligand in **4**.

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(9) The $^{18}OH_2$ was 97% ^{18}O. For **2-O**: m/z 1031 ($M + 2-BF_4-NCCH_3$), ^{18}O = 93%; 1003 ($M + 2-BF_4-NCCH_3-CO$), ^{18}O = 73%. For **1-O**: m/z 1003 ($M + 2-BF_4-2NCCH_3$), ^{18}O = 85%. For **3-O**: m/z 1059 ($M + 2-BF_4$), ^{18}O = 77%, 1003 ($M + 2-BF_4-2CO$), ^{18}O = 13%. All label percentages are ±10%. The magnitude of the error is due to inherent limitations in the FAB method of mass spectral analysis. The $^{13}C\{^1H\}$ NMR spectrum of **3-O** exhibits a δ 164.1 (t, CO) signal and an upfield (4.5-Hz) triplet of ~1.4:1 relative intensity. In addition, a small shoulder is visible to the upfield side of the δ 167.6 singlet (CO_2Me).

(10) Although the carbonyl ligands in **3** do not exchange oxygen with $^{18}OH_2$ at 23 °C, at 74 °C oxygen-18 does exchange into the carbonyl ligands, but not into the ester substituents.

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Supplementary Material Available: Full spectroscopic and analytical data for **3** (1 page). Ordering information is given on any current masthead page.

Use of a Double-Half-Filter in Two-Dimensional ^{1}H Nuclear Magnetic Resonance Studies of Receptor-Bound Cyclosporin

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Linear and cyclic polypeptides with up to approximately 30 amino acid residues include a variety of natural compounds with highly interesting control functions, for example, as hormones or, in the system discussed in this paper, as immunosuppressants. Because of their inherent flexibility, this class of molecules has been elusive to experimental determination of the three-dimensional structure in the unbound state, and because of intrinsic difficulties in both sample preparation and analysis of experimental data, very little knowledge has been accrued on the conformation of the receptor-bound molecules. In this paper we demonstrate the use of a recently described editing technique for ^{1}H NMR spectra, heteronuclear double-half-filters,[2,3] as a basis for detailed studies of the three-dimensional structure of cyclosporin A (CsA)[1] bound to cyclophilin. In addition the technique enables systematic investigations of the intermolecular contacts with the receptor. The cyclophilin-CsA complex is very stable (K_d = 10^{-8} M) and has a molecular weight of 19 200. The experimental approach described here should be generally applicable with stable complexes of comparable size.

^{1}H NMR spectroscopy in solution is by now a generally acceptable method for the determination of the three-dimensional structure of small proteins at atomic resolution.[4] For larger proteins with molecular weights above 10 000-15 000, spectral overlap in the ^{1}H NMR spectra tends to become a limiting factor. In response, a variety of experimental procedures have been proposed to simplify complex ^{1}H NMR spectra with the use of isotope labeling with ^{13}C and ^{15}N, and selective observation of protons bound to these isotopes.[5-9] This approach is particularly attractive for studies of complexes formed between two or more different molecules, since it is conceptually straightforward to label one of the components before formation of the complex. With the use of an X(ω_1,ω_2)-double-half-filter,[2,3] the editing of the ^{1}H NMR spectra can be extended to obtain a subspectrum of the isotope-labeled ligand that allows data collection for a structure determination without interference from the resonances of the much bigger receptor molecule.

In the pulse sequence used (Figure 1), the delay τ is chosen as $\tau = 1/[4^1J(^{13}C,^1H)]$. Application or omission of the individual ^{13}C 180° editing pulses applied simultaneously with the 180° ^{1}H refocusing pulses leads to a total of four different recordings, which are stored separately. Suitably chosen linear combinations of these four recordings yield four subspectra with the desired contents (see text below and Table I). Compared to the corresponding experiment without the double-half-filter, the sensitivity of the experiment in Figure 1 is reduced by a factor $e^{-4\tau/T_2}$, with $\tau = 1/4^1J(^{13}C,^1H)$. The present experiments demonstrate that this is tolerable with molecular weights of up to at least 20 kD.

Table I. Resonance Lines Contained in the Four Subspectra of Figure 2

subspectrum	linear combination[a]	filter pass characteristics[b]
(I) ^{13}C(ω_1)-^{13}C(ω_2) doubly filtered	a + b + c + d	diagonal peaks of and cross peaks between unlabeled protons of cyclophilin
(II) ^{13}C(ω_1)-^{13}C(ω_2) doubly selected	a - b - c + d	diagonal peaks of and cross peaks between ^{13}C-bound protons of CsA
(III) ^{13}C(ω_1)-selected/ ^{13}C(ω_2)-filtered	a - b + c - d	cross peaks manifesting intermolecular NOEs between unlabeled protons of cyclophilin and ^{13}C-bound protons of CsA
(IV) ^{13}C(ω_1)-filtered/ ^{13}C(ω_2)-selected	a + b - c - d	

[a] See caption to Figure 1. [b] Lists all the ^{1}H resonance lines seen in the individual subspectra.

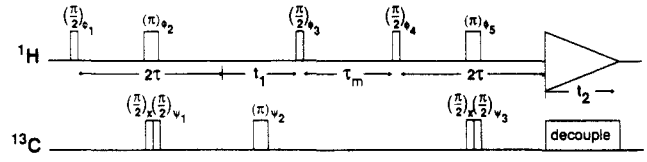

Figure 1. Experimental scheme for ^{1}H NOESY with a ^{13}C(ω_1,ω_2)-double-half-filter, with heteronuclear decoupling during the evolution and detection periods. The phases ϕ_1 to ϕ_5 and ψ_2 are independently alternated between x and $-x$, which results in a phase cycle of 64 steps. The receiver phase is inverted whenever the phase of a $(\pi/2)(^1H)$ pulse is alternated. This basic phase cycle is repeated four times with the following four combinations of ψ_1 and ψ_3: (a) $\psi_1 = \psi_3 = x$; (b) $\psi_1 = -x$, $\psi_3 = x$; (c) $\psi_1 = x$, $\psi_3 = -x$; (d) $\psi_1 = \psi_3 = -x$. The desired subspectra are obtained as linear combinations of combinations a-d (Table I).

In the presently studied system, uniformly 99% ^{13}C labeled CsA (MW = 1265) was bound to the unlabeled protein cyclophilin (MW = 17 900), which is its presumed cellular receptor[10] and is identical with peptidyl-prolyl *cis-trans* isomerase (EC 5.2.1.8).[11] CsA is an immunosuppressive cyclic undecapeptide that has found widespread use in the treatment of allograft rejection following organ transplantations.[12] Figure 2 shows different regions of the four subspectra obtained from a single ^{1}H NOESY experiment with a ^{13}C double-half-filter recorded with the experiment of Figure 1. A survey of the resonance lines contained in each of the four subspectra is afforded by Table I, and experimental details are given in the figure caption.

Of prime interest is the ^{13}C(ω_1)-^{13}C(ω_2) doubly selected subspectrum (II in Figure 2). It contains exclusively diagonal peaks of and NOE cross peaks between protons belonging to CsA. These can thus be analyzed without interference from the background of the receptor resonances. As an illustration the chemical shifts

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(1) Abbreviations and symbols used: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy; ppm, parts per million; CsA, cyclosporin A.

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