Ron New of the University of California, Riverside Mass Spectrometry Center, for obtaining the mass spectral analyses and Johnson-Matthey for a loan of precious metals.

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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Received July 24, 1990

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.

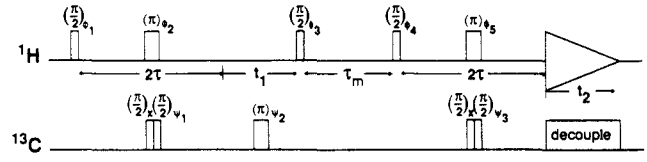

Figure 1. Experimental scheme for ^{1}H NOESY with a ^{13}C(ω_1,ω_2)-double-half-filter, with heteronuclear decoupling during the evolution and detection periods. The phases ϕ_1 to ϕ_5 and ψ_2 are independently alternated between x and $-x$, which results in a phase cycle of 64 steps. The receiver phase 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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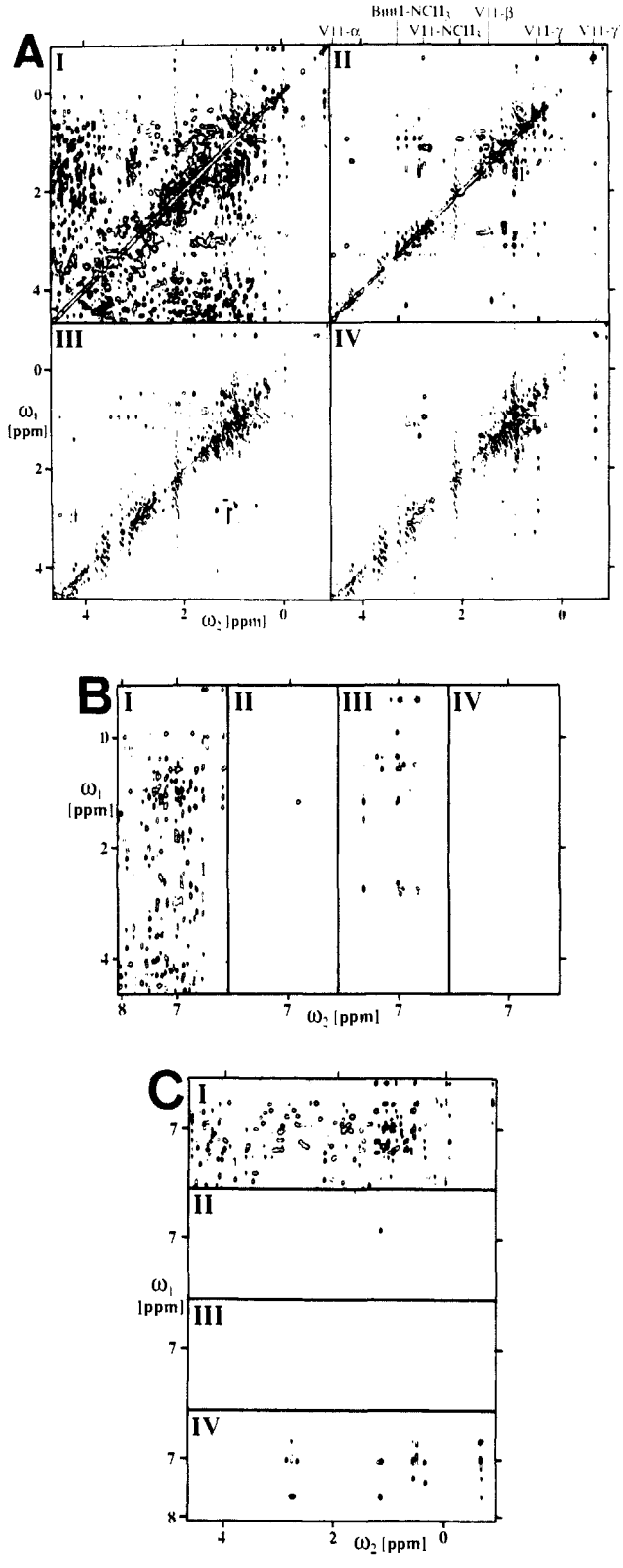

Figure 2. Three spectral regions from a ^1H NOESY spectrum of the cyclophilin complex with ^{13}C-labeled CsA recorded at 500 MHz with a mixing time of 80 ms using the $^{13}C(\omega_1,\omega_2)$-double-half-filter experiment of Figure 1 (complex concentration 0.7 mM, solvent D_2O, pD = 6.0, T = 26 °C, τ = 3.6 ms, t_{1max} = 29 ms, t_{2max} = 127 ms). The following subspectra are shown (see also Table I): (I) $^{13}C(\omega_1)$-$^{13}C(\omega_2)$ doubly filtered; (II) $^{13}C(\omega_1)$-$^{13}C(\omega_2)$ doubly selected; (III) $^{13}C(\omega_1)$-selected/$^{13}C(\omega_2)$-filtered; (IV) $^{13}C(\omega_1)$-filtered/$^{13}C(\omega_2)$-selected. (A) Aliphatic region. In II, the chemical shift of Val 11 $C^{\gamma}H_3$ of CsA is indicated and its NOEs are identified, where Bmt1-NCH_3 stands for the *N*-methyl group of butenylmethylthreonine 1. (B) Region containing the cross peaks between the aromatic region along ω_2 and the aliphatic region along ω_1. (C) The same as B, except that the aromatic region is along ω_1.

of selected resonances in Figure 2A are indicated and some cross peaks with these resonance lines are identified in the spectrum. This subspectrum can be used to study the NOE buildup[4] in ^{13}C-labeled cyclophilin-bound CsA (the proton spin relaxation is significantly influenced by the presence of the ^{13}C spins) and to collect a set of NOE distance constraints as the experimental basis for establishing sequence-specific ^1H resonance assignments and preparing the input for a three-dimensional structure calculation.[4]

Subspectra I, III, and IV in Figure 2 represent a source of information for further characterization of the receptor-ligand complex. In the subspectrum I, all the diagonal and cross peaks originate exclusively from the unlabeled cyclophilin. It corresponds to a conventional ^1H NOESY spectrum of a complex formed between cyclophilin and perdeuterated CsA and, thus, contains all the information needed for a structure determination of liganded cyclophilin. Comparison of this spectrum with that of free cyclophilin can be used to identify the ^1H resonance lines with significant chemical shift changes upon CsA binding. Subspectra III and IV in Figure 2 contain only intermolecular ^1H-^1H NOE cross peaks between cyclophilin and CsA. Once sequence-specific assignments for the receptor-bound ligand are available from spectrum II (Figure 2), these spectra provide the basis for identification of the sites on the ligand that are in contact with the receptor. If in addition sequence-specific ^1H NMR assignments are available also for the receptor protein (subspectrum I in Figure 2A), subspectra III and IV provide direct information on the intermolecular contacts in the receptor-ligand complex.

It is an additional advantage of subspectra III and IV that the diagonal peaks are suppressed.[2,3] As a consequence, the spectra contain only few perturbations from t_1 artifacts and have a flat base plane. Ideally, the diagonal in these subspectra should be completely absent. Residual diagonal peak intensities, such as those seen in Figure 2A, may arise from imperfections in the pulse sequence[2] and from instrumental instabilities. At the same time, strong cross peaks from subspectrum II may also appear as weak signals in subspectra III and IV (in the experiment of Figure 2, we estimated that such leakage occurs to the extent of ca. 5% of the peak intensity in subspectrum II). With the necessary care, such spurious peaks can be identified on the grounds that while subspectrum II is symmetric, subspectra III and IV are asymmetric with respect to the diagonal.

In conclusion, this paper illustrates the potentialities of heteronuclear double-half-filters as a technique enabling conformational studies of receptor-bound ligands in systems that would otherwise be too complex for detailed investigation by ^1H NMR. The experiments provide supplementary data that should eventually be sufficient to characterize complete systems of receptor protein and bound ligand.

Acknowledgment. Financial support by the Schweizerischer Nationalfonds (Project 31-25174.88) is gratefully acknowledged. We thank Mrs. E. Huber for the careful processing of the manuscript.

Vibrational Dynamics of the Cis Peptide Group

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Received August 6, 1990

While the trans peptide group predominates in the stable polypeptide chain of proteins, some non-proline cis peptide groups are found[1] and the less stable cis isomer is observed in aqueous

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