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Identification of solvent-exposed regions of an FK-506 analog, ascomycin, bound to FKBP using a paramagnetic probe

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SUMMARY

The solvent-exposed regions of [U-^{13}C]ascomycin when bound to its putative target protein, FKBP, have been identified based on the different proton longitudinal relaxation rates ($R_1 = 1/T_1$) measured in the absence and presence of the paramagnetic relaxation reagent, 4-hydroxy-2,2,6,6-tetramethyl-piperidinyl-1-oxy (HyTEMPO). The proton T_1s of bound ascomycin were determined using a pulse sequence (T_1-HMQC) which consists of a 180° proton pulse and a variable delay (τ) followed by a heteronuclear multiple quantum correlation (HMQC) experiment. The solvent-exposed regions of ascomycin determined by these experiments are compared to NOE data in which ascomycin/FKBP contacts were identified and to the X-ray structure of the FK-506/FKBP complex.

INTRODUCTION

Ascomycin, originally discovered as an antifungal antibiotic (Arai et al., 1962), is structurally similar to the immunosuppresant FK-506 (Fig. 1). Like FK-506, ascomycin is a potent immunosuppresant (Hatanaka et al., 1988) which binds tightly to the FK-binding protein (FKBP), a peptidyl-prolyl *cis-trans* isomerase (Harding et al., 1989; Siekierka et al., 1989; Maki et al., 1990; Standaert et al., 1990). It has been hypothesized (Harding et al., 1989; Siekierka et al., 1989) that the binding of FK-506, and therefore likely ascomycin to FKBP plays an important role in mediating its immunosuppressive effects. Therefore, we have been studying the 3D structure of the ascomycin–FKBP complex by NMR spectroscopy. In a recent study, we have determined the conformation of ascomycin when bound to FKBP from an analysis of NOEs observed in heteronuclear 3D NOE spectra of ^{13}C-labeled ascomycin bound to FKBP (Petros et al., 1991). We

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FK-506 R= $CH_2CH{=}CH_2$
Ascomycin R= CH_2CH_3 (35 36)

Fig. 1. Structures of FK-506 and ascomycin.

have also identified NOEs between ascomycin and the protein which indicated those portions of ascomycin that interact with FKBP. This kind of information that allows those regions of a ligand that interact with the protein to be distinguished from those portions that are exposed to solvent may be very important for designing improved analogs. For example, with this information it

Fig. 2. Exponential fit of cross-peak intensities, I, measured from a series of T_1-HMQC spectra as a function of the T_1 delay, τ for three ascomycin protons, (A) 18s, (B) 25-Me, and (C) 4r. T_1-HMQC spectra were acquired on a sample of [U-^{13}C]ascomycin bound to FKBP in the absence of HyTEMPO (●) and in the presence of 4 mM (■) and 10 mM (▲) HyTEMPO. A three-parameter fit was carried out to take into account the relatively short value used for the relaxation delay.

may be possible to modify the ligand in such a way that the physical properties are improved or the toxicity is decreased without altering binding affinity (Fesik, 1989).

Recently, we introduced a technique to probe the solvent-exposed regions of bound ligands using the relaxation agent 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy (HyTEMPO) (Fesik et al., 1991). The technique relies upon the change in longitudinal relaxation rate ($R_1 = 1/T_1$) of the solvent-exposed protons of a bound ^{13}C-labeled ligand in the presence of HyTEMPO. The method complements previously described (Arseniev et al., 1986; Petros et al., 1990) approaches for identifying the surface residues of proteins without the loss in sensitivity observed in proton 2D COSY spectra of large molecules. In our case, the T_1s for the individual protons of the ligand are measured in the absence and presence of the paramagnetic reagent using a pulse sequence (T_1-HMQC) which consists of a 180° proton pulse and a variable delay (τ) followed by a heteronuclear multiple quantum correlation (HMQC) experiment (Müller, 1979). In this communication we have applied this technique to delineate those portions of ascomycin that are exposed to solvent when bound to its putative target protein, FKBP.

RESULTS AND DISCUSSION

Proton and ^{13}C assignments were determined for [U-^{13}C]ascomycin bound to FKBP from an analysis of 2D ^{1}H/^{13}C and ^{13}C/^{13}C correlation spectra and 3D NOE data as described elsewhere (Petros et al., 1991). The individual proton T_1s of ascomycin were determined from an exponential fit of the cross-peak volumes measured as a function of τ in the T_1-HMQC experiment. The quality of the data is illustrated in Fig. 2 for three of the ascomycin protons (A, 18s; B, 25-Me; C, 4r) at a HyTEMPO concentration of 0, 4 and 10 mM. An example of the T_1-HMQC spectra obtained in this study is shown in Fig. 3. As can be seen from this spectrum and the exponential fits shown in Fig. 2, HyTEMPO clearly produces differential effects on the bound ascomycin signals. For example, in the absence of HyTEMPO (Fig. 3A) all of the cross peaks are positive with the exception of 17-Me which at this τ value is at the null. At the same τ, in the presence of 4 mM HyTEMPO (Fig. 3B), some of the cross peaks have been severely attenuated, 35a,b, 33r and 34r, or inverted (17-Me) while others are only marginally affected (e.g. 11, 25, 30s and 12r).

A more complete picture is given in Table 1 which lists the proton longitudinal relaxation rates ($R_1 = 1/T_1$) for ascomycin bound to FKBP in the absence and presence of 4 mM and 10 mM HyTEMPO. Also shown in Table 1 is the paramagnetic contribution (R_{1p}) to the total longitudinal relaxation rate at each HyTEMPO concentration. This is simply the difference between the relaxation rate in the presence and absence of HyTEMPO (Niccolai et al., 1982). As can be seen, the largest effects were observed on ascomycin protons in the regions 18-24 and 32-34, and for the ethyl group, position 35-36, while the piperidine ring protons (2-6) were least affected.

As expected, the ascomycin relaxation rates are larger at 10 mM HyTEMPO than 4 mM HyTEMPO. However, for the most part, the relative order of the R_{1p} values for the bound ascomycin protons remains about the same, suggesting that either concentration of HyTEMPO could be used to determine the solvent-exposed region of the bound ligand. The choice of HyTEMPO concentration to use in the experiment will depend on the size of the complex. Since paramagnetic-induced relaxation is brought about predominantly by dipole–dipole interactions between electronic and nuclear spins (Solomon and Bloembergen, 1956), it follows that the slower motions, both rotational and translational, of a larger protein–ligand complex will be more effective in modulating electron–nucleus interactions compared to a smaller complex.

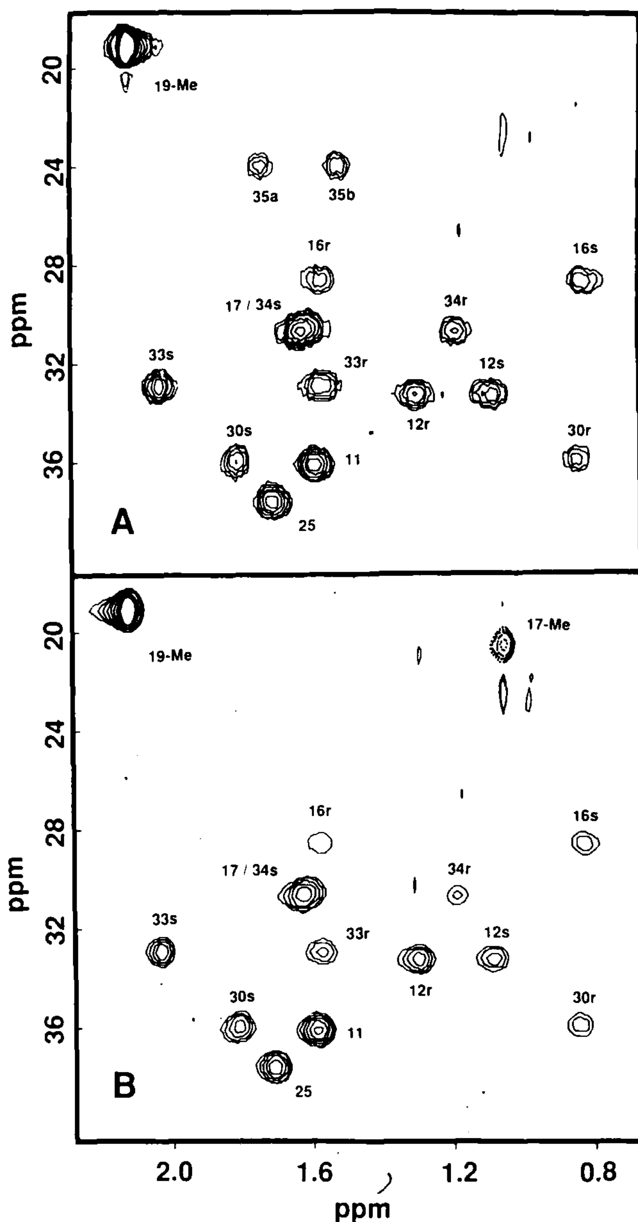

Fig. 3. Sections from T_1-HMQC spectra of [U-^{13}C]ascomycin bound to FKBP. (A) no HyTEMPO. (B) 4 mM HyTEMPO. The spectra were acquired on a Bruker AMX600 spectrometer at 30°C using a sweep width of 21,793.13 Hz in ω_1 and 10,000 Hz in ω_2. 160 complex t_1 points were acquired per experiment with 32 scans per t_1 point, a 2.5-s recycle delay between each scan, and a delay (τ) of 200 ms. Initial spectra, recorded with $\tau = 0$, were phased positive and successive spectra were processed with the same phase constants. In the figure, negative cross peaks are represented with dotted contours. The NMR sample was ~3 mM FKBP/[U-^{13}C]ascomycin in ^2H_2O solution with 50 mM potassium phosphate, 100 mM sodium chloride and 5 mM dithiothreitol-d_{10}, pH 6.5. HyTEMPO was added to this solution as μl volumes of a concentrated stock solution. All methylene protons of ascomycin have been stereospecifically assigned (to be described elsewhere) with the exception of those attached to carbon 35. Methylene protons are thus distinguished using 'r' (pro-r) or 's' (pro-s) descriptors.

TABLE 1
PROTON LONGITUDINAL RELAXATION RATES[a] FOR [U-^{13}C]ASCOMYCIN BOUND TO FKBP IN THE PRESENCE AND ABSENCE OF HyTEMPO

Ascomycin protons	0 mM	4 mM		10 mM	
	R_1 [s^{-1}]	R_1 [s^{-1}]	(R_{1p} [s^{-1}])	R_1 [s^{-1}]	(R_{1p} [s^{-1}])
18s	1.10	2.63	(1.53)	5.56	(4.46)
35a	2.04	3.45	(1.41)	5.88	(3.84)
35b	2.00	3.23	(1.23)	5.56	(3.56)
17-Me	2.86	4.00	(1.14)	5.26	(2.40)
23s	0.72	1.58	(0.86)	4.17	(3.45)
21	0.93	1.79	(0.86)	4.00	(3.07)
20	1.03	1.89	(0.86)	4.00	(2.97)
19-Me	0.68	1.41	(0.73)	3.23	(2.55)
33s	0.76	1.47	(0.71)	3.85	(3.09)
36	1.30	2.00	(0.70)	3.57	(2.27)
23r	0.68	1.37	(0.69)	2.86	(2.18)
33r	0.82	1.47	(0.65)	3.70	(2.88)
32	0.57	1.18	(0.61)	2.63	(2.06)
34r	0.65	1.25	(0.60)	3.13	(2.48)
15	0.98	1.52	(0.54)	2.33	(1.35)
24	0.61	1.11	(0.50)	2.22	(1.61)
25-Me	0.97	1.45	(0.48)	2.56	(1.59)
13-OMe	0.60	1.02	(0.42)	2.08	(1.48)
15-OMe	0.58	0.99	(0.41)	2.04	(1.46)
31	0.60	1.01	(0.41)	1.89	(1.29)
16r	0.90	1.30	(0.40)	2.27	(1.37)
28	0.60	1.00	(0.40)	1.64	(1.04)
31-OMe	0.56	0.93	(0.37)	1.82	(1.26)
14	0.70	1.06	(0.36)	1.96	(1.26)
29	0.66	1.00	(0.34)	1.85	(1.19)
25	0.63	0.95	(0.32)	1.69	(1.06)
12s	0.66	0.95	(0.29)	1.72	(1.06)
12r	0.67	0.94	(0.27)	1.79	(1.12)
13	0.69	0.95	(0.26)	1.56	(0.87)
26	0.70	0.96	(0.26)	1.45	(0.75)
30s	0.68	0.93	(0.25)	1.69	(1.01)
11-Me	1.14	1.37	(0.23)	1.69	(0.55)
27-Me	0.50	0.72	(0.22)	1.33	(0.83)
6r	0.70	0.91	(0.21)	1.37	(0.67)
2	0.70	0.90	(0.20)	1.16	(0.46)
16s	0.91	1.10	(0.19)	1.69	(0.78)
11	0.82	1.01	(0.19)	1.52	(0.70)
3r	0.65	0.81	(0.16)	1.00	(0.35)
5r	0.62	0.75	(0.13)	0.99	(0.37)
3s	0.66	0.78	(0.12)	0.98	(0.32)
4s	0.68	0.79	(0.11)	0.99	(0.32)
5s	0.66	0.71	(0.05)	1.02	(0.36)
4r	0.72	0.76	(0.04)	1.11	(0.39)

[a] Proton longitudinal relaxation rates (R_1) are equal to $1/T_1$. R_{1p} is the paramagnetic contribution to the relaxation rate and is equal to $R_1 - R_1$(HyTEMPO), where R_1(HyTEMPO) is the longitudinal relaxation rate in the presence of HyTEMPO (Niccolai et al., 1982). The ascomycin protons are ordered according to decreasing R_{1p} at 4 mM HyTEMPO. T_1 values were calculated from an exponential fit of the ^{13}C/^{1}H cross-peak volumes measured from a series of T_1-HMQC experiments acquired with 7 different τ values (0, 50, 100, 150, 200, 400, and 600 ms). Experimental details are given in the caption to Fig. 2. Due to overlap of 6s with 18r and 17 with 34s, the T_1s for these four protons were not calculated.

Figure 4 depicts the NMR-derived conformation of ascomycin when bound to FKBP (Petros et al., 1991). The protons attached to filled and checkered carbon atoms were found to be close to FKBP as evidenced by NOEs between these protons and the protein. Most of these ascomycin protons were only marginally affected by 4 mM HyTEMPO. However, a few that displayed NOEs to the protein were also strongly affected by HyTEMPO (e.g., 17-Me and 19-Me). This result suggests that although these ascomycin protons are in contact with FKBP, they are not deeply buried within the protein but are accessible to the spin label. Protons attached to those carbon atoms filled with the jagged lines showed no NOEs to the protein, and the relaxation rates of these protons were also strongly affected by HyTEMPO, suggesting that they are solvent-exposed. It is interesting to note the differential effect of HyTEMPO on the two protons at position 16 (inset Fig. 4 and Table 1). H16r is more strongly affected by HyTEMPO than H16s. This is consistent with the solution structure of bound ascomycin (Petros et al., 1991) in which H16s points towards the buried piperidine ring (2-6) and H16r is oriented towards the solvent-exposed portion of ascomycin. Thus, it is possible to detect quite subtle differences in solvent accessibility using this method.

Recently, an X-ray crystal structure of the FKBP/FK-506 complex was reported (Van Duyne

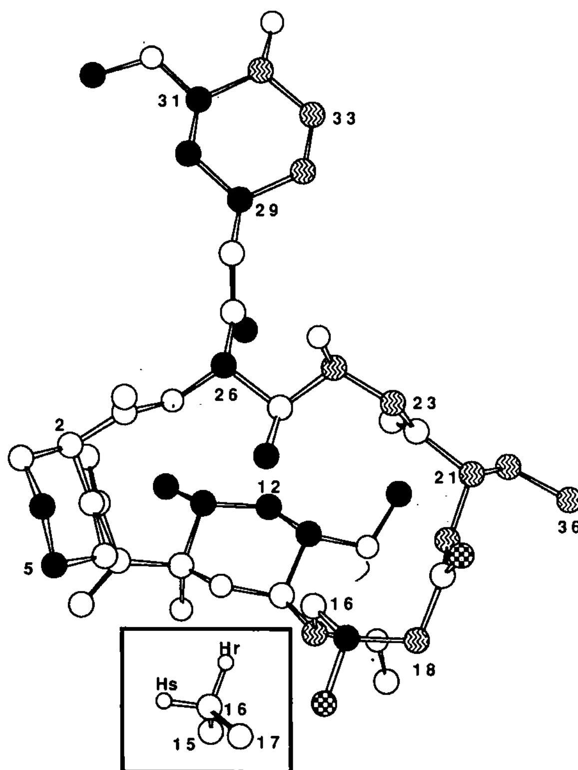

Fig. 4. Three-dimensional structure of ascomycin bound to FKBP. Ascomycin/FKBP NOEs were observed from ascomycin protons attached to the filled and checkered carbon atoms (Petros et al., 1991). The protons attached to the jagged and checkered carbon atoms exhibited the largest ($R_{1p} \geqslant 0.5$) paramagnetic contribution to the relaxation rate upon addition of HyTEMPO (4 mM).

et al., 1991). The X-ray structure shows roughly 50% of the ligand surface being buried at the protein–ligand interface with the regions around the allyl group (analogous to the ethyl group of ascomycin) and the cyclohexyl ring being exposed to the solvent. Consistent with the X-ray crystallographic results, we have found that those protons in the ethyl group region (18-24, 35-36) as well as the cyclohexyl ring protons (32-34) are most affected by HyTEMPO (Table 1). In addition, those portions of ascomycin that were the least affected by the addition of HyTEMPO (e.g., piperidine ring protons) were the most deeply buried regions of FK-506 in the X-ray crystal structure (Van Duyne et al., 1991).

CONCLUSIONS

In summary, we have identified the solvent-exposed regions of ascomycin when bound to its putative receptor, FKBP, from the selective change in proton longitudinal relaxation rates caused by the addition of a paramagnetic probe. The approach that we have used assumes that there are no specific interactions between HyTEMPO and the complex. Otherwise, a distorted view of the exposed surface of the complex may be obtained. In our case, no chemical shift changes were observed for the ascomycin protons upon the addition of HyTEMPO nor were any of the ascomycin resonances broadened beyond detection, suggesting that a specific interaction between HyTEMPO and the inhibitor is unlikely. The method used to measure the T_1s of bound ^{13}C-labeled ligands is highly sensitive, easy to implement, and can yield important structural information on enzyme–inhibitor complexes prior to the determination of a complete solution structure. This information on the solvent-exposed regions of enzyme-bound ligands can be helpful for identifying those portions of the ligand that can be modified without affecting the binding to its receptor and should therefore be a valuable aid in drug design.

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