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NMR studies of [U-^{13}C]cyclosporin A bound to human cyclophilin B

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NMR data (^{1}H and ^{13}C chemical shifts, NOEs) on [[U-^{13}C]cyclosporin A bound to cyclophilin B were compared to previously published data on the [U-^{13}C]CsA/CyPA complex [Fesik et al., (1991) Biochemistry 30, 6574–6583]. Despite only 64% sequence identity between CyPA and CyPB, the conformation and active site environment of CsA when bound to CyPA and CyPB are nearly identical as judged by the similarity of the NMR data.

Cyclophilin B; Cyclosporin A; NMR; Conformation

1. INTRODUCTION

The cyclophilins (CyPs) are a family of proteins found in mammalian, fungal, and procaryotic organisms [1–12] that catalyze *cis-trans* peptidyl-prolyl isomerization [13,14]. Cyclophilin from human T cells (CyPA) as well as other cyclophilins, bind to and are inhibited by the immunosuppressive drug cyclosporin A (CsA) (Fig. 1) [1–12]. For most CsA analogs, the relative affinity for cyclophilin correlates with their immunosuppressive activity, suggesting that the immunosuppressive effects of CsA involve binding to cyclophilin [1,15–18]. However, for a few CsA analogs cyclophilin binding does not correlate with immunosuppressive activity (e.g. [MeAla^6] and [MeBm$_2$t^{1}CsA), questioning the role of cyclophilin in CsA-mediated immunosuppression [17,18].

One possible explanation for these apparent discrepancies is that CsA exerts its immunosuppressive effects by binding to a different, yet structurally related, cyclophilin. Recently, a second human cyclophilin, cyclophilin B (CyPB) [19], has been cloned and was found to be 64% identical to human CyPA. Unlike CyPA which is cytosolic, CyPB contains a hydrophobic N-terminal signal sequence which is removed upon expression in *E. coli* [19]. Recent localization studies have shown CyPB to be located in the endoplasmic reticulum, Golgi and vesicles in the cytoplasm (Jin, et al., unpublished). CyPB possesses *cis-trans* peptidyl-prolyl isomerase (PPIase) activity which is inhibited by CsA [19] at three-fold higher concentration than CyPA. Thus, CyPB may also play an significant role in T-cell activation.

In this report we describe NMR studies on the binding of [U-^{13}C]CsA to human cyclophilin B and compare the results to previously described NMR studies on the [U-^{13}C]CsA/CyPA complex [20].

Abbreviations: CyP, cyclophilin (A or B); CsA, cyclosporin A; NOE, nuclear Overhauser effect; HSQC, heteronuclear single quantum correlation; HMQC-NOESY, heteronuclear multiple quantum correlation-nuclear Overhauser effect spectroscopy.

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2. MATERIALS AND METHODS

2.1. *Materials*

[U-^{13}C]CsA was isolated from *Beauveria nivea* ATCC 34921 (American Type Culture Collection; Rockville, MD) culture containing [U-^{13}C]Celtone medium (Martek Corp., Columbia, MD) [20]. Recombinant human cyclophilin A was prepared as recently described [21].

A genetically truncated human cyclophilin B (residues 24–208) was overexpressed in *E. coli* and purified to homogenity as previously described [19].

2.2. *NMR experiments*

The cyclophilin samples (A and B) were exchanged into a D_2O solution (pH 6.5) containing phosphate buffer (50 mM), NaCl (100 mM), and dithiothreitol (5 mM) and concentrated to ~1.0 mM with a centricon YM-10 (Amicon).

The [U-^{13}C]CsA/cyclophilin (A and B) complexes were prepared by gently shaking a suspension of ~1.5 mol equivalents of [U-^{13}C]CsA in a D_2O solution of cyclophilin at 6°C for 12 h under argon. The excess CsA was removed by centrifugation.

All NMR spectra were acquired on a Bruker AMX500 NMR spectrometer at 20°C. The spectra were processed using in-house written software on Silicon Graphics computers or the Bruker UXNMR software package on a Bruker X32.

The heteronuclear single quantum correlation (HSQC) spectra [22] were acquired using 2048 complex points in F_2 and 512 complex points in F_1, with 96 scans. The sweep width was 7812.5 Hz in F_2 and 7462.7 Hz in F_1.

The 3D HMQC-NOESY experiments [23] were acquired as 48(t_1)

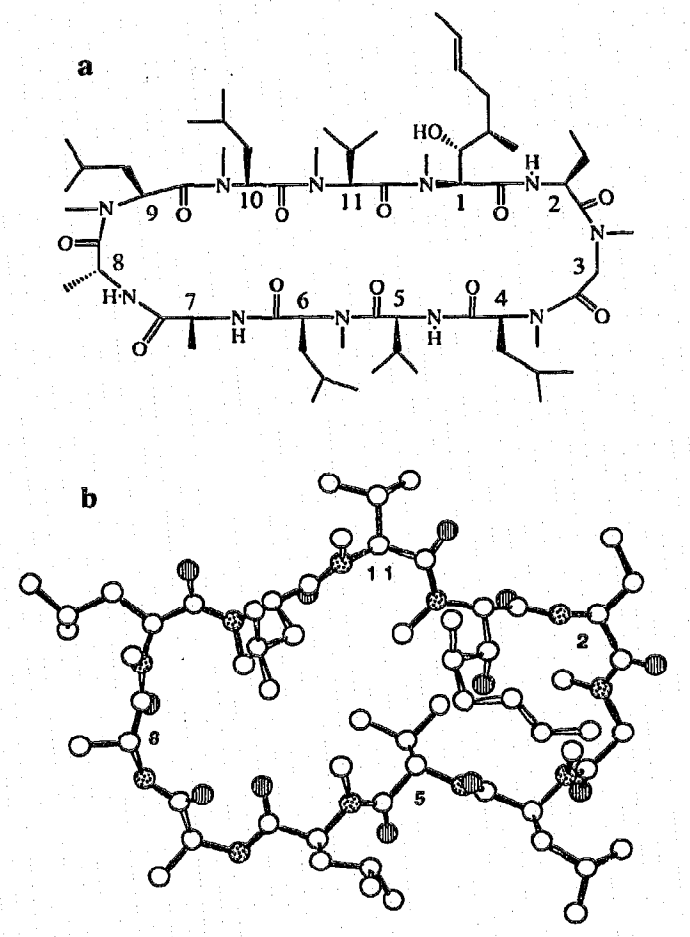

Fig. 1. (a) Structure of CsA. (b) Conformation of CsA when bound to CyPA [20].

× 128 (t_2) × 2048(t_3) complex points using a mixing time of 50 ms and spectral widths of 7462.7 Hz in ω_1 and 7812.5 Hz in ω_2 and ω_3. 32 acquisitions were collected per t_3 experiment for a total experimental time of approximately 6 days.

3. RESULTS

As a first step in probing for any structural differences between the [U-^{13}C]CsA/CyPA and [U-^{13}C]CsA/CyPB complexes, we compared their proton NMR spectra. As expected for two proteins with 64% sequence identity, the proton NMR spectra of the two CsA/CyP complexes, which mainly consisted of protein signals (data not shown), were markedly different. In contrast, as shown in the two-dimensional heteronuclear single quantum correlation (HSQC) spectra [22] of [U-^{13}C]CsA bound to CyPA (Fig. 2a) and CyPB (Fig. 2b), the ^{1}H and ^{13}C chemical shifts of [U-^{13}C]CsA when bound to either protein are nearly identical. The assignment of the signals was easily deduced by a direct comparison of the two HSQC spectra and confirmed in the 3D HMQC-NOESY spectra [20]. The observed chemical shift differences in ppm ($\delta_{CypB}-\delta_{CypA}$) were: 1H$^\beta$ (−0.04), 5H$^\alpha$ (−0.07), 8H$^\alpha$ (−0.05), 9H$^\alpha$ (−0.10), 9H$^\beta$ (−0.16, −0.06), 9C$^\beta$ (−0.3), 11NCH3 (+0.05), 11H$^\alpha$ (−0.04), 11C$^\alpha$ (+0.4). All other signals resonate at the same frequencies within the limits of experimental error [20]. In Fig. 2 some signals are invisible at the level chosen for the plot, but can be observed with lower contouring. The residual protein cross-peaks that are detected in the HSQC spectra (indicated by P in Fig. 2) are different in the two cases, reflecting the sequence differences in the two proteins.

In Fig. 3 three-dimensional HMQC-NOESY spectra [23] are compared for [U-^{13}C]CsA bound to CyPA (top of each panel a–e) and CyPB (bottom of each panel a–e). ^{1}H, ^{1}H planes were extracted at the same ^{13}C frequencies in the two data sets. As shown by the comparison of the spectra obtained for the two complexes, similar NOEs, with the same relative intensities, were observed. Some weak NOEs present in the CypA dataset could not be observed in the 3D NOE spectrum of CypB due to a lower signal-to-noise ratio.

In Fig. 1b the conformation of CsA when bound to CyPA is shown that had been previously determined from 3D NOE data [20]. The bound conformation of CsA [20,24,25] was found to be very different from the previously determined NMR-derived or X-ray crystal structure of cyclosporin A determined in the absence of cyclophilin [26]. When bound to cyclophilin, CsA adopts a *trans* 9,10 peptide bond [24] in contrast to the 9,10 *cis* peptide bond found in free CsA [26]. In addition, the backbone conformation and the orientations of some of the side chains greatly differ between the structures [20,25].

It is important to note key long range NOEs important in defining the overall backbone conformation of CsA when bound to CyPA (Fig. 1b) [20] were also observed in the 3D NOE spectrum of the CsA/CyPB complex. Some of these include NOEs from 6NCH$_3$ to 5H$^\alpha$, 1NCH$_3$ and 10NCH$_3$ (Fig. 3a) and NOEs from 5Hγ^2 to 1NCH$_3$ and 3NCH$_3$ (Fig. 3b). NOEs between CsA protons indicative of a particular side chain conformation were also found to be the same in the CsA/CyPA [20] and CsA/CyPB complexes. For example, the α- proton of MeBmt^1 is close to its own β and δCH$_3$ side chain protons (Fig. 3c) and MeLeu^6(H$^{\delta 2}$) is in close proximity to MeLeu^6(H$^\alpha$) (Fig. 3b) in both complexes. NOEs between CsA and the protein were also found to be similar in the two complexes (e.g. Fig. 3d). An exception is shown in Fig. 3e in which an additional NOE between MeVal^{11} (NCH$_3$) and an aromatic proton of the protein (arrow) was observed in the [U-^{13}C]CsA/CyPB complex. Another difference is shown in Fig. 2d in which a small change in chemical shift of one of the NOE peaks involving the protein and MeVal^{11}(Hγ^2) was observed, suggesting the CyP binding pocket in the vicinity of the MeVal^{11} CsA residue is slightly different in the two complexes. This is supported by the differences in chemical shift of the 11NCH3, 11H$^\alpha$, and 11C$^\alpha$ signals of CsA when bound to CyPA or CyPB.

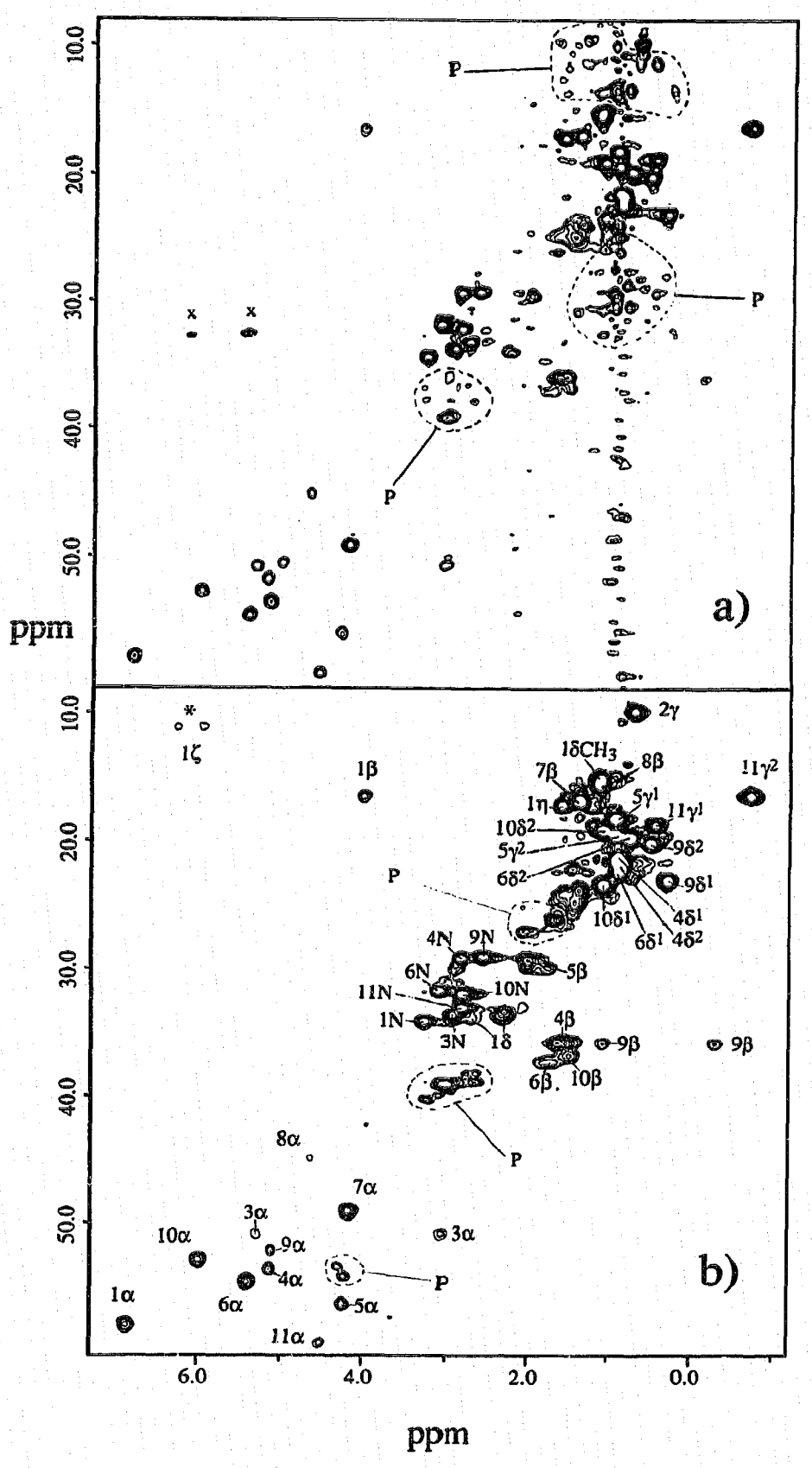

Fig. 2. Contour plots of the HSQC spectra of [U-^{13}C]CsA bound to (a) CyPA and (b) CyPB. The 'x' refers to experimental artifacts. *The MeBmt^{1}(H^{ζ}) signals were not fully ^{13}C-decoupled at the power level employed.

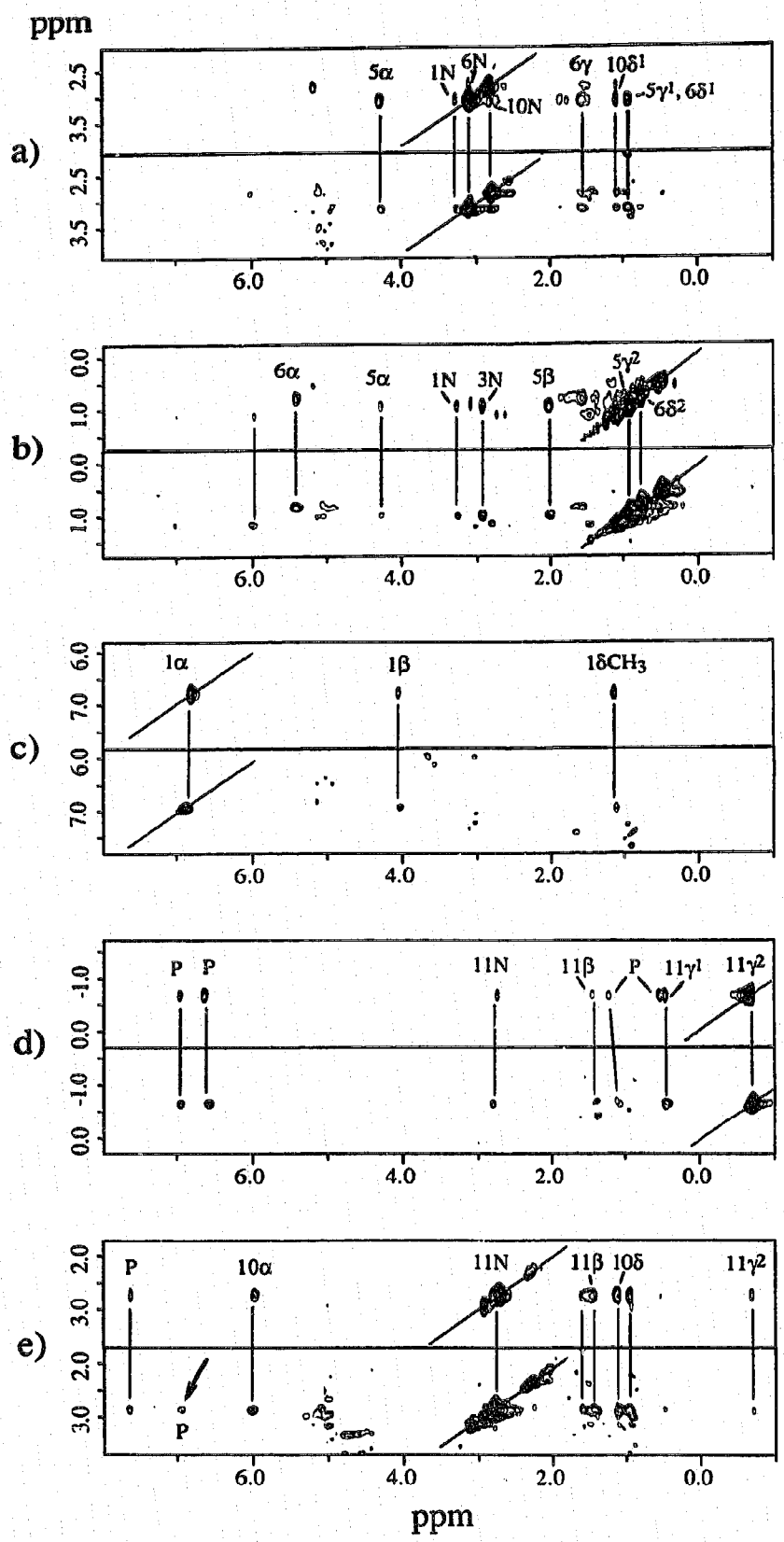

Fig. 3. Comparison of corresponding cross-sections (ω_2, vertical axis; ω_3, horizontal axis) from 3D-HMQC-NOESY spectra of [U-^{13}C]CsA bound to CyPA (top of each panel) and CyPB (bottom of each panel), acquired with a mixing time of 50 ms. In both spectra the planes were taken at the same ^{13}C chemical shifts (ω_1) of (a) 31.8, (b) 19.4, (c) 58.1, (d) 16.3, (e) 33.2 ppm. The skewed solid lines indicate the diagonal peaks on the ^{1}H–^{1}H NOESY planes.

4. DISCUSSION

Despite only 64% sequence identity between CyPA and CyPB, the chemical shift and NOE data suggest that the conformation and active site environment of CsA when bound to CyPA and CyPB is very similar. Even for CsA residues (e.g. residue 1 and 6) in which structural changes produced analogs in which cyclophilin binding and immunosuppressive activity did not correlate [17,18], the chemical shifts and NOEs involving these residues were found to be identical. These results suggest that the apparent discrepancies in CyP binding and immunosuppressive activity displayed by some CsA analogs cannot simply be explained by invoking CyPB as the biologically relevant cyclophilin. Indeed, the relative amounts of PPIase inhibition for CyPA and CyPB with [MeAla^6] and [MeBm$_2$t^1]CsA show little difference with the two cyclophilin isoforms (Anderson et al., unpublished). It may be that the complexes of cyclophilins (A and/or B) with cyclosporin or CsA analogs may exhibit differential affinities with partner proteins immediately downstream in the signal transduction cascade and that is the relevant immunosuppressive readout.

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