Identification of Solvent-Exposed Regions of Enzyme-Bound Ligands by Nuclear Magnetic Resonance

S. W. Fesik,* G. Gemmecker, E. T. Olejniczak, and A. M. Petros

Pharmaceutical Discovery Division
Abbott Laboratories, Abbott Park, Illinois 60064

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Structural information on enzyme/inhibitor complexes can be valuable for designing clinically useful enzyme inhibitors. In addition to the conformation of the bound ligand, it would be helpful to distinguish between those portions of the ligand that interact with the enzyme from those that are exposed to solvent. This information is important for identifying the functional groups of the ligand that could be modified to improve the physical properties (e.g., water solubility) of the inhibitor without affecting binding affinity.[1]

Approaches for identifying surface residues of proteins and peptides using paramagnetic agents have been proposed.[2-6] Kopple and co-workers have recently shown[6] that the nitroxyl group can be a useful tool for identifying hydrophobic surface residues of proteins from the cancellation of antiphase cross-peak components in two-dimensional correlated (2D COSY) NMR spectra observed in the presence of paramagnetic agents. For larger proteins, however, this method is less effective due to broad protein signals, which result in the poor sensitivity of 2D COSY cross peaks. Furthermore, it is difficult to quantitate the paramagnetic effects using this method since the amount of COSY cross peak component cancellation is dependent on nonuniform $^1H,^1H$ coupling constants.

In this communication we describe an approach for identifying the solvent-exposed regions of enzyme-bound ligands using the paramagnetic relaxation reagent, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy (HyTEMPO). The method is illustrated in the study of the immunosuppressant cyclosporin A (CsA) bound to its putative target protein, cyclophilin.[7]

The approach that we have employed relies on the distance-dependent change in T_1 caused by the nitroxyl group of HyTEMPO.[2] The proton T_1 values of uniformly ^{13}C labeled CsA were measured in the presence and absence of HyTEMPO using a pulse sequence that consists of a 180° proton pulse and variable delay (τ) followed by a heteronuclear multiple quantum correlation (HMQC) experiment.[8] The proton T_1 values were determined from an exponential fit of the $^{13}C/^1H$ cross-peak volumes measured as a function of τ.[9] Using this method, the signals corresponding to ^{13}C-labeled CsA which had been previously assigned[10] were selectively detected in the presence of unlabeled cyclophilin, and many proton T_1 values of CsA could be measured due to the resolution obtained in the 2D experiment.

Table I. Proton Longitudinal Relaxation Rates[a] for [U-^{13}C]Cyclosporin A Bound to Cyclophilin in the Presence and Absence of 5 mM HyTEMPO

CsA protons	R, s^{-1}		
	0 mM	5 mM	R(5 mM)/R(0 mM)
$9NCH_3$	1.30	6.67	5.13
8α	1.54	6.25	4.06
$3\alpha'$	1.09	4.35	3.99
1η	1.16	4.35	3.75
3α	1.20	3.84	3.20
$4NCH_3$	1.22	3.45	2.82
7α	1.47	3.85	2.62
$9\delta^1$	2.78	7.14	2.56
1ϵ	1.43	3.57	2.49
1ξ	1.41	3.33	2.36
$6\delta^2$	1.96	4.17	2.13
$6\delta^1$	2.63	5.56	2.11
4γ	2.50	5.26	2.10
7β	2.17	4.55	2.10
6α	1.41	2.94	2.09
$9\beta'$	1.35	2.70	2.00
$9\delta^2$	1.67	3.33	2.00
4α	2.08	4.00	1.92
6β	1.47	2.78	1.89
$4\beta'$	2.44	4.55	1.86
1δ	2.00	3.70	1.85
6γ	1.64	3.03	1.85
$10\delta^1$	2.04	3.70	1.81
$1\delta CH_3$	2.33	4.17	1.79
4β	2.94	5.26	1.79
2α	1.37	2.38	1.73
9α	1.47	2.50	1.70
$3NCH_3$	1.11	1.89	1.70
1β	1.33	2.17	1.63
5β	1.85	2.94	1.58
9β	0.98	1.51	1.54
$6NCH_3$	1.35	2.08	1.54
10β	1.39	2.13	1.53
1α	1.35	2.04	1.51
$5\gamma^2$	2.04	3.03	1.49
$10\delta^2$	2.50	3.70	1.48
$10NCH_3$	1.56	2.17	1.39
1γ	1.56	2.17	1.39
5α	1.53	2.13	1.39
$1NCH_3$	1.20	1.64	1.37
10α	1.51	2.00	1.32
$5\gamma^1$	3.33	4.35	1.31
$11NCH_3$	1.41	1.78	1.26
11α	1.47	1.82	1.24
2γ	2.33	2.63	1.13
$11\gamma^1$	1.37	1.47	1.07
$11\gamma^2$	2.27	2.33	1.03

[a] Proton longitudinal relaxation rates (R) are equal to $1/T_1$. T_1 values were calculated from an exponential fit of the $^{13}C/^1H$ cross-peak volumes measured from a series of 180(^1H)-τ-HMQC experiments acquired with eight different τ values. The sample for the NMR experiment consisted of a 1.3 mM ^2H_2O solution of the [U-^{13}C]CsA/cyclophilin (1/1) complex prepared as previously described.[10] Two-dimensional NMR spectra were acquired on a Bruker AMX500 (500 MHz) spectrometer at 20 °C using a sweep width of 7463 Hz in ω_1 and 10 000 Hz in ω_2; 32 scans were acquired per τ value using a 2.6 s delay between scans for a total time of 7.5 h per experiment.

In Table I the proton longitudinal relaxation rates ($R = 1/T_1$) of CsA bound to cyclophilin are given in the absence and presence of 5 mM HyTEMPO along with the ratio of the $1/T_1$ values. The effect of the spin label varied widely for different parts of CsA bound to cyclophilin. The largest effects were observed on protons from CsA residues 3, 4, 6, 7, 8, and 9 as well as from the end of the MeBmt[1] side chain. The CsA protons that were the least affected by the addition of HyTEMPO were part of CsA residues 11, 2, and 10. No chemical shift changes were observed for the CsA protons, and none of the CsA signals were broadened beyond detection upon the addition of HyTEMPO. Thus, it is unlikely that a specific interaction between CsA and HyTEMPO occurs.

* To whom correspondence should be addressed.

(1) Fesik, S. W. In *Computer-Aided Drug Design. Methods and Applications*: Perun, T. J., Propst, C. L., Eds.; Marcel Dekker: New York, 1989; pp 133-184. Fesik, S. W. *Nature* **1988**, *332*, 865-866. Fesik, S. W.; Zuiderweg, E. R. P.; Olejniczak, E. T.; Gampe, R. T., Jr. *Biochem. Pharmacol.* **1990**, *40*, 161-167.
(2) Niccolai, N.; Valensin, G.; Rossi, C.; Gibbons, W. A. *J. Am. Chem. Soc.* **1982**, *104*, 1534-1537.
(3) Kopple, K. D.; Zhu, P.-P. *J. Am. Chem. Soc.* **1983**, *105*, 7742-7746.
(4) Lane, A. N.; Jardetzky, O. *Eur. J. Biochem.* **1985**, *152*, 411-418.
(5) Arseniev, A. S.; Sobol, A. G.; Bystrov, V. F. *J. Magn. Reson.* **1986**, *70*, 427-435.
(6) Petros, A. M.; Mueller, L.; Kopple, K. D. *Biochemistry* **1990**, *29*, 10041-10048.
(7) Handschumacher, R. E.; Harding, M. W.; Rice, J.; Drugge, R. J.; Speicher, D. W. *Science* **1984**, *226*, 544-547.
(8) Müller, L. *J. Am. Chem. Soc.* **1979**, *101*, 4481-4484.
(9) The longitudinal relaxation rates ($1/T_1$) reported in Table I were obtained by using eight τ values (0, 50, 100, 150, 200, 300, 600, and 1000 ms). Inspection of the $1/T_1$ values calculated from the initial rates (first four points) did not significantly alter the results, indicating that spin-diffusion effects were minimal.
(10) Fesik, S. W.; Gampe, R. T., Jr.; Eaton, H. L.; Gemmecker, G.; Olejniczak, E. T.; Neri, P.; Holzman, T. F.; Egan, D. A.; Edalji, R.; Simmer, R.; Helfrich, R.; Hochlowski, J.; Jackson, M. *Biochemistry* **1991**, *30*, 6574-6583.

0002-7863/91/1513-7080$02.50/0

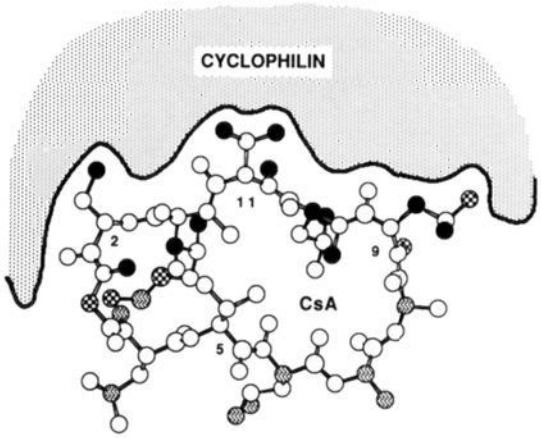

Figure 1. Three-dimensional structure of CsA bound to cyclophilin.[10] CsA/cyclophilin NOEs were observed from CsA protons attached to the filled and checkered carbon atoms.[10] The protons attached to the jagged and checkered carbon atoms exhibited the largest change in relaxation rate [R(5mM)/R(0mM) ≥ 2] upon the addition of HyTEMPO.

Figure 1 depicts the three-dimensional structure of CsA when bound to cyclophilin that was recently determined by NMR.[10] The protons attached to the filled and checkered carbon atoms were found to be in close proximity to cyclophilin, as evidenced by NOEs between these CsA protons and the protein.[10] Most of these protons (e.g., 11γ^2, 11γ^1, 2γ, 11NCH$_3$, 10α) were only marginally affected by the addition of HyTEMPO. However, some of the protons that displayed NOEs to cyclophilin (those attached to the checkered carbon atoms) also exhibited the largest changes in T_1 upon the addition of spin label (9NCH$_3$, 9δ^1, 3α, 3α', 1η, and 1ϵ). This suggests that these CsA protons are not as deeply buried within the protein. A possible explanation is that cyclophilin is located to one side of the CsA protons while the spin label is accessible to the other.

Large effects of the spin label were also observed for the protons attached to the jagged filled carbon atoms (Figure 1), indicating that these CsA residues (4, 6, 7, 8) are exposed to solvent. These results are consistent with the structure/activity relationships of CsA analogues[11-14] in which cyclophilin binding and immunosuppressant activity was found to be relatively insensitive to modifications of these CsA residues. Also consistent with the structure/activity relationships[11-14] was the finding that the CsA residues (1, 2, 9, 10, 11) important for cyclophilin binding and immunosuppressant activity are solvent shielded as indicated by the reduced effect of added HyTEMPO.

In summary, we have described an approach for identifying the solvent-exposed regions of enzyme-bound ligands. The methods are easy to implement and highly sensitive and can rapidly yield important structural information on enzyme/inhibitor complexes. The approach is expected to be especially useful in NMR studies of larger systems in which complete three-dimensional structures may be difficult to obtain.

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Registry No. HyTEMPO, 2226-96-2.

(11) Quesniaux, V. F. J.; Schreier, M. H.; Wenger, R. M.; Hiestand, P. C.; Harding, M. W.; Van Regenmortel, M. H. V. *Eur. J. Immunol.* **1987**, *17*, 1359–1365.
(12) Wenger, R. M. *Prog. Allergy* **1986**, *38*, 46–64.
(13) Wenger, R. M.; Payne, T. G.; Schreier, M. H. *Prog. Clin. Biochem. Med.* **1986**, *3*, 157–191.
(14) Durette, P. L.; Boger, J.; Dumont, F.; Firestone, R.; Frankshun, R. A.; Koprak, S. L.; Lin, C. S.; Melino, M. R.; Pessolano, A. A.; Pisano, J.; Schmidt, J. A.; Sigal, N. H.; Staruch, M. J.; Witzel, B. E. *Transplant. Proc.* **1988**, *20*, 51–57.

Bis[(dimethoxyethane)lithium(I)] 1,2,4,5-Tetrakis(trimethylsilyl)benzenide. The First 6C-8π Antiaromatic Benzene Dianion[1]

Akira Sekiguchi, Keisuke Ebata, Chizuko Kabuto, and Hideki Sakurai*

Department of Chemistry and Organosilicon Research Laboratory, Faculty of Science Tohoku University, Aoba-ku, Sendai 980, Japan
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Cyclic conjugated polyenes exhibit aromaticity if the number of π electrons is $(4n + 2)$, whereas antiaromaticity is predicted for $4n\pi$ electron systems.[2] Thus anions such as cyclopentadienyl anion[3] and cyclooctatetraenyl dianion[4] are aromatic, while the benzene dianion bearing 8π electrons could be antiaromatic.[5] However, a theoretical study on $C_6H_6^{2-}$ suggested that the benzene dianion could be stabilized by distorting the structure into a boat form.[5b] The planar structures with D_{2h} or D_{6h} symmetry were calculated to be 20.2 and 36.6 kcal/mol higher in energy than the distorted one. Very recently, we reported bis[(tetrahydrofuran)lithium(I)] hexakis(trimethylsilyl)benzenide as the first example of such a benzene dianion distorted highly into a boat form with two lithium atoms located on the same side of the benzene ring.[6,7] We report here the synthesis and characterization of bis[(dimethoxyethane)lithium(I)] 1,2,4,5-tetrakis(trimethylsilyl)benzenide as the first 6C-8π antiaromatic system.

Reduction of 1,2,4,5-tetrakis(trimethylsilyl)benzene **1** (367 mg, 1.0 mmol) with lithium metal (60 mg, 8.6 mmol) in dry-oxygen-free dimethoxyethane (DME, 6 mL) at room temperature immediately led to a dark brown solution of the benzene dianion of **1**. The solvent was removed in vacuo, and then dry degassed hexane was introduced by vacuum transfer. Crystallization from hexane afforded dark brown crystals of bis[(dimethoxyethane)-lithium(I)] 1,2,4,5-tetrakis(trimethylsilyl)benzenide **2**, [Li(dme)]$_2$[1,2,4,5-(Me$_3$Si)$_4$C$_6$H$_2$].[8] Reduction of **1** in tetrahydrofuran similarly yielded the dianion; however, suitable crystals for X-ray crystallography were not obtained. The dianion **2** was extremely air- and moisture-sensitive.[9]

The structure of **2** was determined by X-ray crystallography.[10]

(1) Chemistry of Organosilicon Compounds. 288.
(2) Breslow, R. *Angew. Chem.* **1968**, *80*, 573.
(3) Aoyagi, T.; Shearer, H. M. M.; Wade, K.; Whitehead, G. *J. Organomet. Chem.* **1979**, *175*, 21. See also the following reviews. (a) Setzer, W. N.; Schleyer, P. v. R. *Adv. Organomet. Chem.* **1985**, *24*, 353. (b) Schade, C.; Schleyer, P. v. R. *Adv. Organomet. Chem.* **1987**, *27*, 169.
(4) Noordik, J. H.; van den Hark, T. E. M.; Mooij, J. J.; Klaassen, A. A. K. *Acta Crystallogr.* **1974**, *B30*, 833.
(5) (a) Nakayama, M.; Ishikawa, H.; Nakano, T.; Kikuchi, O. *J. Mol. Struct. (Theochem)* **1989**, *184*, 369. (b) Podlogar, B. L.; Glauser, W. A.; Rodriguez, W. R.; Raber, D. J. *J. Org. Chem.* **1988**, *53*, 2127. (c) Jesse, R. E.; Hoytink, G. J. *Chem. Phys. Lett.* **1967**, *1*, 109. See theoretical studies on the interaction between lithium and benzene. (d) Jebli, R.; Volpilhac, G. F.; Hoarau, J.; Achard, F. *J. Chem. Phys.* **1984**, *81*, 13. (e) Morton-Blake, D. A.; Corish, J.; Beniere, F. *Theor. Chim. Acta* **1985**, *68*, 389.
(6) Sekiguchi, A.; Ebata, K.; Kabuto, C.; Sakurai, H. *J. Am. Chem. Soc.* **1991**, *113*, 1464.
(7) Distortion into the boat structure for hexakis(trimethylsilyl)benzene dianion is not due to the steric factor of the bulky trimethylsilyl group. (See footnote 12 of ref 6.)
(8) Compound **2**: dark brown crystals; ^{1}H NMR (300 MHz, toluene-d_8, 233 K) δ 0.21 (s, 36 H, SiMe$_3$), 3.30 (s, 8 H, DME), 3.46 (s, 12 H, DME), 5.11 (s, 2 H, ArH); ^{13}C NMR (75.5 MHz, toluene-d_8, 233 K) δ 2.74, (SiMe$_3$), 60.5 (DME, OMe), 70.7 (DME, OCH$_2$), 80.8 (C$_{ar}$Si), 161.8 (C$_{ar}$H); ^{29}Si NMR (59.6 MHz, toluene-d_8, 233 K) δ −19.2; ^{7}Li NMR (116.6 MHz, toluene-d_8, 233 K) δ 10.7 ppm (from LiCl in MeOH).
(9) The dianion **2** readily reverted to **1** on exposure to air and reacted with H$_2$O to give 1,3,4,6-tetrakis(trimethylsilyl)cyclohexa-1,4-diene and 2,3,5,6-tetrakis(trimethylsilyl)cyclohexa-1,3-diene in 93 and 4% yield, respectively, in addition to a small amount of **1**.
(10) A single crystal (0.3 × 0.3 × 0.3 mm) of **2** was sealed in a capillary glass tube for data collection. Diffraction data were collected at 13 °C on a Rigaku Denki AFC-5R diffractometer with a rotating anode (45 kV, 200 mA) with graphite-monochromatized Mo Kα radiation (λ = 0.71069 Å). A total of 5015 reflections with 2θ = 3°–45° were collected. Crystal data: MF = Si$_4$O$_4$C$_{26}$Li$_2$H$_{58}$; MW = 560.97; monoclinic; a = 10.801 (10), b = 11.087 (6), c = 31.971 (10) Å, β = 97.08° (5); V = 3799 (8) Å^3; space group $P2_1/c$; Z = 4; D_c = 0.980 g/cm^3. The final R factor was 0.056 (R_w = 0.052) for 2375 reflections with $F_o > 3\sigma(F_o)$.

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