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Probing the Molecular Interaction of Chymotrypsin with Organophosphorus Compounds by ^{31}P Diffusion NMR in Aqueous Solutions

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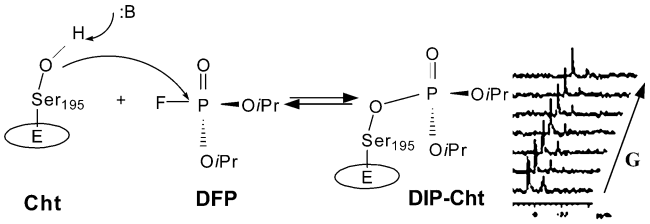

In the present study, we applied for the first time ^{31}P diffusion NMR to resolve different species obtained by the addition of organophosphorus compounds (OP) such as diisopropyl phosphorofluoridate (DFP) or 1-pyrenebutyl phosphorodichloridate (PBPDC) to α-chymotrypsin (Cht). ^{31}P diffusion NMR was used since the products of these reactions constitute a mixture of OP-covalent conjugates of the enzyme and OP-containing hydrolysis products that have noninformative ^{1}H NMR spectra. It was shown that the peak, attributed to the covalent native diisopropylphosphoryl-Cht (DIP-Cht) conjugate by chemical shift considerations, has a greater diffusion coefficient (D = (0.65 ± 0.01) × 10^{-5} cm^2 s^{-1}) than expected from its molecular mass (approximately 25 kDa). This peak was therefore suggested to consist of at least two superimposed signals of diisopropyl phosphoryl (DIP) pools of high and low molecular weights that happen to have the same chemical shift. This conclusion was substantiated by the use of DMSO-d_6 that separated the overlapping signals. Diffusion measurements performed on the extensively dialyzed and unfolded DIP-Cht conjugate still resulted in a high diffusion coefficient ((0.30 ± 0.05) × 10^{-5} cm^2 s^{-1}) relative to the assumed molecular mass. This observation was attributed to a dynamic dealkylation at the OP moiety (i.e., aging) that occurred during the relatively long diffusion measurements, where DIP-Cht was converted to the corresponding monoisopropyl phosphoryl Cht (MIP-Cht) conjugate. Homogeneous aged forms of OP-Cht were obtained by use of DFP and heat-induced dealkylation of DIP-Cht, and by PBPDC that provided the aged form via the hydrolysis of a P-Cl bond (PBP-Cht). The thermally stable aged conjugates enabled a reliable determination of the diffusion coefficients over several days of data acquisition, and the values found were (0.052 ± 0.002) × 10^{-5} cm^2 s^{-1} and (0.054 ± 0.004) × 10^{-5} cm^2 s^{-1} for the MIP-Cht and the PBP-Cht adducts, respectively, values in the range expected for a species with a molecular weight of 25 kDa. The advantages and limitations of ^{31}P diffusion NMR in corroborating the type of species that prevail in such systems are briefly discussed.

Introduction

Many serine hydrolases are inhibited irreversibly by organophosphorus (OP) esters by the formation of a stoichiometric (1:1) covalent conjugate with the active-site serine (Chart 1).[1,2] Depending on the OP moiety and the hydrolase source, the activity of the inhibited enzyme can be regenerated by a variety of nucleophiles.[1b,3] However, for certain OP-inhibited enzymes obtained by

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(1) (a) Hartley, B. S. *Annu. Rev. Biochem.* **1960**, *29*, 45−49. (b) Aldrich, W. N.; Reiner, E. In *Enzyme Inhibitors as Substrates*; Neuberger, A., Tatum, E. L., Eds.; North-Holland Publishing Co.: Amsterdam and London, and American Elsevier Publishing: New York, 1972.

(2) (a) Cohen, J. A.; Oosterbaan, R. A.; Warringa, M. G. P. J.; Jansz, H. S. *Disuss. Faraday Soc*. **1955**, *20*, 114−124. (b) Oosterbaan, R. A.; Kunst, P.; Cohen J. A. *Biochim. Biophys. Acta* **1955**, *16*, 299−307.

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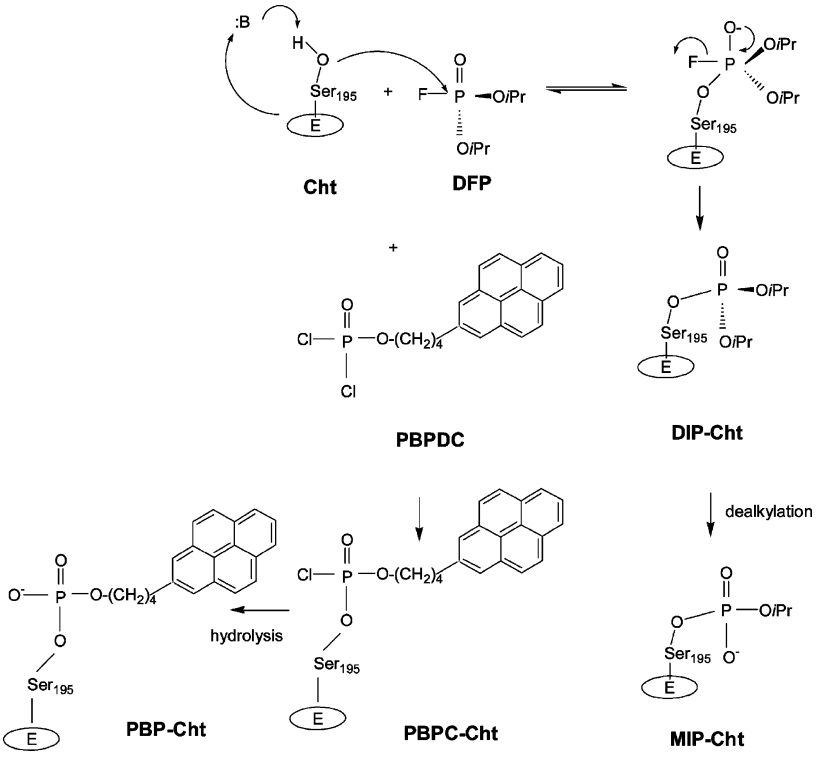
CHART 1



the use of OPs such as *O,O*-diisopropyl phosphorofluoridate (DFP) or *O*-pinacolyl methylphosphonofluoridate (soman), reactivation is hampered by a parallel aging reaction[4] that converts the inhibited enzyme to a nonreactivatable form. In most cases, the aging process is accompanied by detachment of an alkyl group from the OP-bound moiety,[5] a reaction that introduces a negatively charged oxygen on the OP residue (P−O$^-$) (Chart 1).

The detailed structures of aged and nonaged OP-inhibited enzymes were obtained from X-ray crystallography[6] as well as neutron diffraction studies.[7] Proton nuclear magnetic resonance spectroscopy was applied to monitor changes in the catalytic histidine-protonated forms of butyrylcholinesterase[8a] and acetylcholinesterase[8b] following inhibition by an OP. However, since ^{1}H NMR spectroscopy is inadequate for structural analysis of the organophosphoryl moiety of OP-inhibited serine hydrolases, ^{31}P NMR chemical shifts have been used in the past to monitor the kinetics of the aging process and elucidate the structure of aged and nonaged OP conjugates of a variety of serine hydrolases in aqueous solutions.[9]

Diffusion NMR, as obtained by the pulsed field gradient spin- or stimulated-echo sequences, is known to be a useful method for studying the diffusion characteristics and hence the molecular mobility of different systems.[10] Diffusion NMR was also used to map the molecular interactions between different molecular species in supramolecular systems.[11−13] This technique was used to study association constants, ion pairing, self-aggregation, and sizes of different supramolecular systems as well as the structure of many organometallic complexes.[11] More recently, it was used to study hydrogen-bound molecular capsules[12,13] and to probe the interactions between different ligands with proteins and DNA fragments.[14,15] In view of the limited information that one may gain from ^{1}H NMR studies of OP conjugates of serine hydrolases,

(3) Froede, H. C.; Wilson, I. B. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 5, pp 87−114.

(4) (a) Hobbiger, F. *Br. J. Pharmacol.* **1965**, *14*, 73−77. (b) Berends, F.; Postumus, C. H.; Sluys, I. V. D.; Deierkauf, F. A. *Biochim. Biophys. Acta* **1959**, *34*, 576−578.

(5) (a) Michel, O. H. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1958**, *17*, 275−283. (b) Berends, F.; Postumus, C. H.; Sluys, I. V. D.; Deierkauf, F. A. *Biochim. Biophys. Acta* **1959**, *34*, 576−578. (c) Sun, M.; Chang, Z.; Shau, M.; Huang, R.; Chou, T. *Eur. J. Biochem.* **1979**, *100*, 527−530.

(6) (a) Stroud, R. M.; Kay, L. M.; Dickerson, R. E. *J. Mol. Biol*. **1974**, *83*, 185−208. (b) Cohen, J. H.; Silverton, E. W.; Davies, D. R. *J. Mol. Biol.* **1981**, *148*, 449−479. (c) Ringe, D.; Mottonen, J. M.; Gelb, M. H.; Abeles, R. H. *Biochemistry.* **1986**, *25*, 5633−5638. (d) Harel, M.; Su, C. T.; Frolow, F.; Ashani, Y.; Silman, I.; Sussman, J. L. *J. Mol. Biol.* **1991**, *221*, 909−918. (e) Millard, C. B.; Kryger, G.; Ordentlich, A.; Greenblat, H. M.; Harel, M.; Raves, M. L.; Segall, Y.; Barak, D.; Shafferman, A.; Silman, I.; Sussman, J. L. *Biochemistry* **1999**, *38*, 7032−7039. (f) Millard, C. B.; Koellner, G.; Ordentlich, A.; Shafferman, A.; Silman, I.; Sussman, J. L. *J. Am. Chem. Soc*. **1999**, *121*, 9883−9884.

(7) Kossiakoff, A. A.; Spencer, S. A. *Nature (London)* **1980**, *288*, 414−416

(8) (a) Viragh, C.; Harris, T. K.; Reddy, P. M.; Massiah, M. A.; Mildvan, A. S.; Kovach, I. M. *Biochemistry* **2000**, *39*, 16200−16205. (b) Messiah, M. A.; Viragh, C.; Reddy, P. M.; Kovach, I. M.; Johnson, J.; Rossenberry, T. L.; Mildvan, A. S. *Biochemistry* **2001**, *40*, 5682−5690

(9) (a) Gorenstein, D. G.; Findlay, J. B. *Biochem. Biophys. Res. Commun.* **1976**, *72*, 640−645. (b) van Der Drift, A. C. M.; Beck, H. C.; Dekker: W. H.; Hulst, A. G.; Wils, E. R. J. *Biochemistry.* **1985**, *24*, 6894−6903. (c) Gorenstein, D. G.; Shah, D.; Chen, R.; Kallick, D. *Biochemistry.* **1989**, *28*, 2050−2058. (d) Grunwald, J.; Segall, Y.; Shirin, E.; Waysbort, D.; Steinberg, N.; Silman, I.; Ashani, Y. *Biochem. Pharmacol.* **1989**, *19*, 3157−3168. (e) Oleksyszyn, J.; Powers, J. C.; *Biochemistry* **1991**, *30*, 485−493. (f) Kovach, I. M.; Mckay, L.; Vander Velde, D. *Chirality* **1993**, *5*, 143−149. (g) Segall, Y.; Waysbort, D.; Barak, D.; Ariel, N.; Doctor, B. P.; Grunwald, J.; Ashani, Y. *Biochemistry.* **1993**, *32*, 13441−13450.

(10) (a) Stejskal, E. O.; Tanner, J. E. *J. Chem. Phys.* **1965**, *42*, 288−292. (b) Tanner, J. E. *J. Chem. Phys.* **1970**, *52*, 2523−2526. For reviews concerning the application of the pulsed-gradient NMR techniques to different systems see: c) Stilbs, P. *Prog. NMR Spectrosc.* **1987**, *19*, 1−45. (d) Price, W. S. *Concepts Magn. Reson.* **1997**, *9*, 299−336. (e) Johnson, C. S., Jr. *Prog. NMR Spectrosc.* **1999**, *34*, 203−256.

we examined the use of ^{31}P diffusion NMR to study the products array of the reaction of α-chymotrypsin (Cht) with DFP and 1-pyrenebutyl phosphorodichloridate (PBPDC). Cht, being a globular enzyme of molecular weight of approximately 25 kDa, is phosphorylated by DFP and PBPDC at the active-site serine, and the reaction products offer a convenient model system for the application of diffusion NMR to characterize the interaction of OPs with serine hydrolases in aqueous solutions. We thought to verify which of the ^{31}P peaks arise from covalent binding to the protein, identify OP-containing ligands that associate reversibly with the enzyme (i.e., noncovalent interactions), and use the covalent adduct as a probe for measuring the diffusion coefficient of Cht itself.

Experimental Section

General Methods. All chemical reagents and solvents used were as purchased. Bovine pancreatic α-chymotrypsin (Type II, 3× crystallized and salt-free lyophilized) was obtained from Sigma Chemical Co.

Sample Preparation. DIP-Cht Conjugate. A 0.1 mL portion of DFP stock solution (0.57 M in 2-propanol) was added dropwise to a 5 mL Cht solution (4 mM) in Hepes buffer (50 mM, pH 7.8), and the mixture was incubated for 24 h at room temperature. The loss of enzymatic activity was monitored using the chromogenic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide.[16] To remove traces of unreacted DFP and low molecular weight hydrolysis products, the inhibited enzyme was dialyzed for 24 h at 4 °C against 50 mM of Hepes buffer, pH 7.22 (filtration cutoff of 15 kDa).

MIP-Cht Conjugate. The aged form, MIP-Cht, was obtained by heat-accelerated dealkylation of the nonaged conjugate DIP-Cht. Briefly, 3.5 mL of dialyzed DIP-Cht obtained as described above was incubated at 40 °C for 4 days. Based on ^{31}P NMR chemical shifts, the native aged form was accompanied with a small amount of OP-containing species that was assumed to represent the thermally denaturated MIP-Cht.

PBP-Cht Conjugate. This adduct was obtained by slow addition of 30 mg of PBPDC in 15 mL of acetonitrile to a solution of 200 mg of Cht in 200 mL of Hepes buffer (50 mM, pH 7.8). The reactants were mixed at room temperature for 20 h. Following centrifugation to remove solid particles, the solution was dialyzed as described above against 10 L of distilled water. The dialysis was carried out for 12 days with three exchanges of the distilled water and lyophilized to form a white solid of the PBP-Cht conjugate. For diffusion measurements, 42.9 mg of the PBP-Cht were dissolved in 0.55 mL of D_2O.

Unfolding. Unfolding of OP-Cht conjugates was carried out by either adding solid guanidine hydrochloride (Gd·HCl) or dilution of the concentrated stock solutions to produce the desired final concentration of Gd·HCl. As judged from the chemical shift changes, unfolding was completed after 2 h at room temperature.

Diffusion NMR. NMR diffusion measurements were performed on a 500 MHz Avance Bruker NMR spectrometer equipped with a 5 mm inverse probe with gradient set capable of generating magnetic field pulse-gradients (in the *z*-direction) of about 50 G cm^{-1}. Diffusion experiments were performed using the stimulated echo diffusion sequence.[10b] The pulsed gradients were incremented from 0.5 to 35 G cm^{-1} in seven steps, and their duration varied from 4 to 12 ms in the different diffusion experiments. The pulse gradient separation was 70 ms. The relaxation delay was 3.05 s, and the number of transients was optimized to obtain an acceptable signal-to-noise ratio (SNR). All measurements were repeated at least three times, and the reported diffusion coefficients are the mean ± standard deviation of three experiments. Only data where the correlation coefficients of $\ln(I/I_0)$ versus $\gamma^2\delta^2g^2(\Delta - \delta/3)$, often abbreviated as b values, were higher than 0.990 are reported. The measurements were all performed at 25 °C, and a capillary with hexamethyl phosphoroamidate (HMPA) was used as an external standard to ascertain the stability of the generated gradients throughout the data acquisition time. The ^{31}P NMR chemical shifts were referenced to external trimethyl phosphate (TMP) that was set to 0 ppm.

Results

The addition of DFP to chymotrypsin afforded a ^{31}P NMR spectrum which was consistent with the one previously reported.[9g] To better characterize the peak at −3.2 ppm (relative to TMP), we thought of using diffusion NMR. It was assumed that this peak would reflect the diffusion coefficient of a covalently OP-bound moiety to Cht. Figure 1 shows the stackplot of the stimulated echo diffusion experiment of the DFP-inhibited Cht.

Surprisingly, despite the substantial differences in the molecular weights of DFP (184) and DIP-Cht (25 kDa), not very different diffusion coefficients of $(0.93 \pm 0.07) \times 10^{-5}$ and $(0.65 \pm 0.01) \times 10^{-5}$ cm^2 s^{-1} were extracted for the free DFP and the DIP-Cht adduct, respectively, from these diffusion experiments (Figure 2). Two possible explanations may account for the above observations: (1) the peak at −3.2 ppm does not represent the covalent DIP-Cht adduct or (2) the peak is a superposition of at least two species that have the same chemical shift but differ in their diffusion coefficients. Based on the extensive NMR work previously performed on the above system,[9d,g] the second explanation appears more reasonable.

A closer look at the spectra shown in Figure 1, especially at the high gradient values, reveals that in fact

(11) For a few selected examples, see: (a) Rymdén, R.; Carlfors, J.; Stilbs, P. *J. Inclusion Phenom.* **1983**, *1*, 159−167. (b) Mayzel, O.; Cohen, Y. *J. Chem. Soc., Chem. Commun.* **1994**, 1901−1902. (c) Cohen, Y.; Ayalon, A. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 816−818. (d) Mayzel, O.; Gafni, A.; Cohen, Y. *Chem. Commun.* **1996**, 911−912. (e) Gafni, A.; Cohen, Y. *J. Org. Chem.* **1997**, *62*, 120−125. (f) Cameron, K. S.; Fielding, L. *J. Org. Chem.* **2001**, *66*, 6891−6895. (g) Pochapsky, S. S.; Mo, H.; Pochapsky, T. C. *J. Chem. Soc., Chem. Commun.* **1995**, 2513−2514. (h) Mo, H.; Pochapsky, T. C. *J. Phys. Chem. B* **1997**, *101*, 4485−4486. (i) Zuccaccia, C.; Bellachioma, G.; Cardaci, G.; Macchioni, A. *Organometallics* **2000**, *19*, 4663−4665. (j) Valentini, M.; Rügger, H.; Pregosin, P. S. *Helv. Chim. Acta* **2001**, *84*, 2833−2853. (k) Avram, L.; Cohen, Y. *J. Org. Chem.* **2002**, *67*, 2639−2644. (l) Hori, A.; Kumazawa, K.; Kusukawa, T.; Chand, D. K.; Fujita, M.; Sakamoto, S.; Yamaguchi, K. *Chem. Eur. J.* **2001**, *7*, 4142−4149. (m) Kotch, F. W.; Sidorov V.; Lam Y.-F.; Kayser K. J.; Hojun L.; Kaucher M. S.; Davis J. T. *J. Am. Chem. Soc.* **2003**, *125*, 15140−15150.

(12) (a) Frish, L.; Matthews, S. E.; Böhmer, V.; Cohen, Y. *J. Chem. Soc., Perkin Trans. 2* **1999**, 669−671. (b) Frish, L.; Vysotsky, M. O.; Matthews, S. E.; Böhmer, V.; Cohen, Y. *J. Chem. Soc., Perkin Trans. 2* **2002**, 88−93. (c) Frish, L.; Vysotsky, M. O.; Böhmer, V.; Cohen, Y. *Org. Biomol. Chem.* **2003**, *1*, 2011−2014.

(13) (a) Avram, L.; Cohen, Y. *J. Am. Chem. Soc.* **2002**, *124*, 15148−15149. (b) Avram, L.; Cohen, Y. *Org. Lett.* **2002**, *4*, 4365−4368. (c) Avram, L.; Cohen, Y. *Org. Lett.* **2003**, *5*, 3329−3332. (d) Avram, L.; Cohen, Y. *J. Am. Chem. Soc.* **2003**, *125*, 16180−16181.

(14) (a) Jones, J. A.; Wilkins, D. K.; Smith, L. J.; Dobson, C. M. *J. Biomol. NMR* **1997**, *10*, 199−203. (b) Mansfield, S. L.; Jayawickrama, D. A.; Timmons, J. S.; Larive, C. K. *Biochim. Biophys. Acta* **1998**, *1382*, 257−265. (c) Zhang, W.; Smithgall, T. E.; Gmeiner, W. H. *Biochemistry* **1998**, *37*, 7119−7126. (d) Price, W. S.; Tsuchiya, F.; Arata, Y. *J. Am. Chem. Soc.* **1999**, *121*, 11503-1151. (e) Chang, X.; Keller, D.; O'Donoghue, S. I.; Led, J. J. *FEBS. Lett.* **2002**, *515*, 165−170. (f) Weljie, A. M.; Yamniuk, A. P.; Yoshino, H.; Izumi, Y.; Vogel, H. J. *Protein Sci.* **2003**, *12*, 228−236.

(15) (a) Lapham, J.; Rife, J. P.; Moore, P. B.; Crothers, D. M. *J. Biomol. NMR* **1997**, *10*, 255−262. (b) Gmeiner, W. H.; Hudalla, C. J.; Soto, A. M.; Marky, L. *FEBS. Lett.* **2000**, *465*, 148−152. (c) Yang, X.; Sanghvi, Y. S.; Gao, X. *J. Biomol. NMR* **1997**, *10*, 383−388.

(16) Delmar, E. G.; Largman, C.; Brodrick, J. W.; Geokas, M. C. *Anal. Biochem.* **1979**, *99*, 316−320.

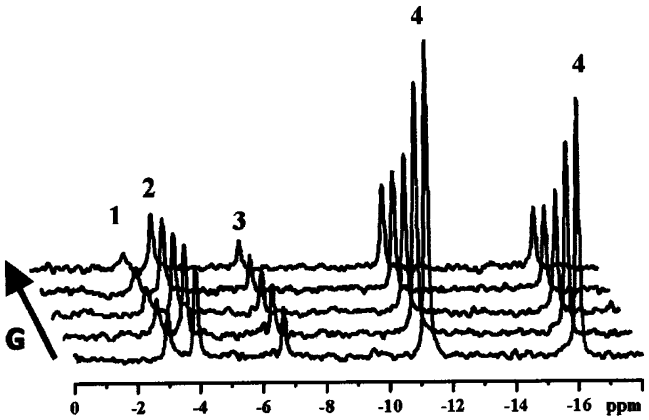


FIGURE 1. ^{31}P NMR (202 MHz, 25 °C, D_2O) spectra as a function of the gradient strength (G) for the products array obtained by reacting DFP and Cht (before dialysis and unfolding): (1) the assumed DIP-Cht adduct; (2) diisopropyphosphoric acid (DIP); (3) triisopropyl phosphate (TIP); (4) DFP.

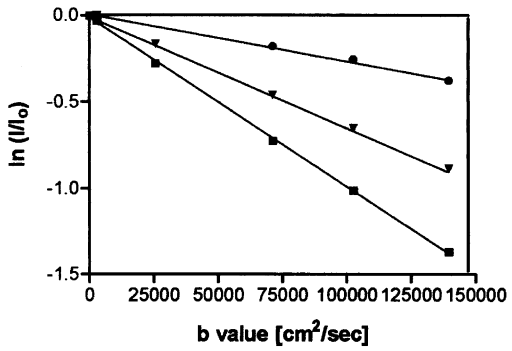


FIGURE 2. Natural log of the normalized signal attenuation (ln I/I_0) as a function of the b values for peaks of DFP (■) and the peaks attributed to the DIP-Cht adduct before (▼) and after dialysis and unfolding (●).

beneath the relatively sharp peak ($\Delta\nu_{1/2} = 40$ Hz) at −3.2 ppm, there is a less intense broad peak ($\Delta\nu_{1/2} \sim 200$ Hz) that was not sampled in the shown diffusion experiment. Indeed, dialysis of this particular sample resulted in the disappearance of the sharp signal leaving only the broad component for which we could not measure the diffusion coefficient due to its low SNR and its relatively short T2. To further verify the composite nature of the signal at −3.2 ppm, we titrated the sample shown in Figure 1 by DMSO-d_6 as shown in Figure 3. Addition of 100 μL of DMSO-d_6 produced a moderate splitting of the peak (Figure 3B) that was more apparent after the addition of an additional 100 μL of DMSO-d_6 (Figure 3C). Thus, the conclusion reached from the diffusion experiments was corroborated using DMSO-d_6. Again, the poor SNR and T2 did not allow measurement of the diffusion coefficients of these peaks.

To estimate the diffusion coefficient of the broad peak that remained after dialysis, a partially unfolded DIP-Cht adduct was obtained by the addition of 1 M Gd·HCl. As expected, unfolding produced a much sharper peak (at −3.6 ppm) that could be analyzed by ^{31}P diffusion NMR. Figure 2 shows the normalized signal decays as a function of the diffusion weighting for free DFP and the peak attributed to the covalent Cht-DIP conjugate, before and after dialysis and unfolding. It is clear that the

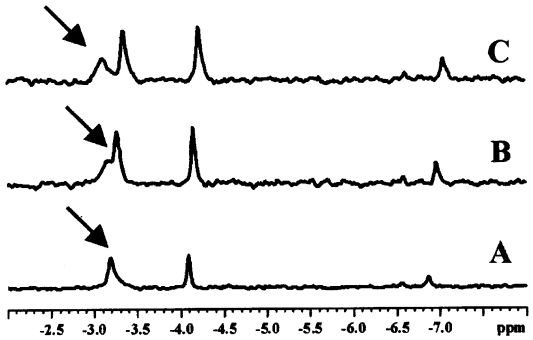


FIGURE 3. Sections of the ^{31}P NMR spectra (202 MHz, 25 °C, D_2O) of peak attributed to the DIP-Cht adduct (A) before, and after addition of (B) 100 μL and (C) 200 μL of DMSO-d_6.

diffusion coefficient obtained for this sample, after extensive dialysis and partial unfolding ($(0.30 \pm 0.05) \times 10^{-5}$ cm^2 s^{-1}), is significantly smaller than that found for this peak prior to dialysis. Yet, this value is still greater than the expected diffusion coefficient for a molecular species of about 25 kDa. Inspection of the spectra of these diffusion experiments (data not shown) clearly demonstrate that the unfolded DIP-Cht adduct underwent time-dependent changes during the relatively long diffusion measurements (72 h), and partial decomposition that resulted in the biased disappearance of the monitored signal not due to diffusion. Therefore, the value of $(0.30 \pm 0.05) \times 10^{-5}$ cm^2 s^{-1} is only a reflection of the high limiting value of the diffusion coefficient for the covalent DIP-Cht adduct.

To further substantiate these results, the MIP-Cht adduct that is the more stable aged product of DIP-Cht was obtained by incubating the dialyzed solution of DIP-Cht at 40 °C for 4 days (Chart 1). Aging was confirmed by demonstrating the complete resistance to reactivation in the presence of 3-(hydroxyiminomethyl)-1-methylpyridinium iodide (3-PAM)[9d,17] as opposed to the DIP-Cht conjugate that could be reactivated, in part, by 3-PAM. Following unfolding of MIP-Cht in 6 M Gd·HCl, diffusion measurements with different diffusion weightings were performed. When measurements were carried out with a pulse gradient length (δ) of 4 ms we could hardly see any signal decay for the MIP-Cht adduct. However, diffusion experiments with a δ of 7 and 12 ms, using an external reference of HMPA to ascertain the stability of the gradient, were quite informative (Figures 4 and 5).

The diffusion coefficient extracted from this set of diffusion experiments was $(0.052 \pm 0.002) \times 10^{-5}$ cm^2 s^{-1}, which more is in the range expected for species having a molecular mass of 25 kDa.[18]

The diffusion measurements were repeated with a different form of an aged conjugate, PBP-Cht (Chart 1). Initially, this adduct was selected for attempting proton NMR spectroscopy to obtain an independent measure of

(17) Cohen, D.; Erlanger, B. F. *J. Am. Chem. Soc.* **1960**, *82*, 3928−3934.

(18) Previous ^{1}H NMR diffusion studies performed on the monomeric complex of the calcium-binding protein myosin light chain two (MLC2) with a detergent, and whose molecular weight was estimated to be about 23.5 kDa, was found to be $\sim 0.108 \times 10^{-5}$ cm^2 s^{-1}; see: Dingley, A. J.; Mackay, J. P.; Shaw G. L.; Hambly, B. D.; King, G. F. *J. Biomol. NMR* **1997**, *10*, 1−8. Dingley, A. J.; Mackay, J. P.; Morris, M. B.; Chapman, B. E.; Kuchel, P. W.; Hambly, B. D.; King, G. F. *J. Biomol. NMR* **1995**, *6*, 321−328.

TABLE 1. Diffusion Coefficients and the Chemical Shifts of DFP and the Different OP-Cht Adducts

system	diffusion coefficients (cm ^{2} s^{-1})	chemical shifts[a] (ppm)
free DFP	$(0.93 \pm 0.07) \times 10^{-5}$	−13.8
DIP-Cht before dialysis and unfolding	$(0.65 \pm 0.01) \times 10^{-5}$	−3.2
DIP-Cht after dialysis and partial unfolding	$(0.30 \pm 0.05) \times 10^{-5}$	−3.6
MIP-Cht (aged) after complete unfolding	$(0.052 \pm 0.002) \times 10^{-5}$	−4.0
PBP-Cht (aged) after complete unfolding	$(0.054 \pm 0.004) \times 10^{-5}$	−2.8

[a] Chemical shifts are referenced to external trimethyl phosphate (TMP).

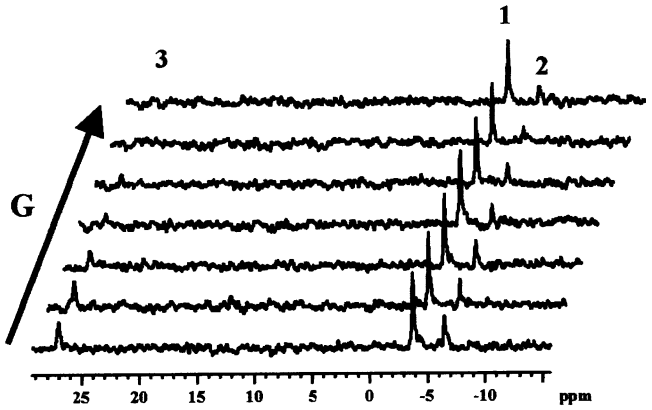


FIGURE 4. ^{31}P NMR (202 MHz, 25 °C, D_2O) spectra as a function of the gradient strength (G) for the unfolded MIP-Cht conjugates obtained by thermal aging of the DIP-Cht adduct: (1) MIP-Cht; (2) denaturated adduct; (3) HMPA.

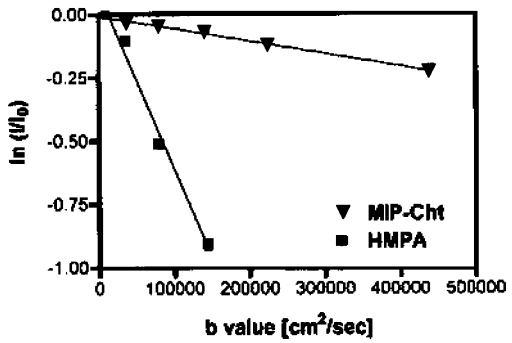


FIGURE 5. Natural log of the normalized signal attenuation ($\ln I/I_0$) as a function of the b values for the peaks of MIP-Cht (▼) and HMPA (■).

the diffusion coefficient. However, the ^{1}H NMR spectrum was found to be noninformative for this purpose. ^{31}P diffusion measurements with long δ (12 ms), and the same external reference of HMPA, resulted in a diffusion coefficient of $(0.054 \pm 0.004) \times 10^{-5}$ cm^2 s^{-1}, that is in excellent agreement with the calculated value for MIP-Cht. The diffusion coefficients of the different systems and adducts along with their chemical shifts are summarized in Table 1.

Discussion

We have demonstrated that ^{31}P diffusion NMR is indeed a useful tool for characterizing enzyme−adduct arrays in systems where the ^{1}H NMR spectra are noninformative because of relatively short T_2. These diffusion measurements, which could be performed with a standard inverse probe using a conventional high resolution NMR spectrometer, clearly demonstrated that the peak seen at −3.2 ppm is composed of two pools of diisopropyl phosphates that happen to have the same chemical shift. Thus, ^{31}P diffusion NMR unequivocally demonstrated that the DIP moiety in the protein-bound and the "free" OP residue have the same chemical shift in the aqueous solution but differ significantly in their diffusion coefficients. The separation of the overlapped peaks by DMSO is consistent with this conclusion.

The ^{31}P NMR signal of the OP-Cht covalent adducts enabled, for the first time, the determination of the diffusion coefficient of the enzyme itself. In fact, any NMR active nuclei can serve as such a probe, and recently, ^{19}F diffusion NMR was used to measure the interaction of two small inhibitors of elastase.[19] In these NMR diffusion experiments, however, due to the noncovalent nature of the association of the enzyme with the inhibitors and the fast exchange between the bound and free ligands, the diffusion coefficient of the enzymes themselves were not determined.

High-resolution diffusion NMR offers an efficient tool for the screening of ligand−protein reversible interactions without the need for ligands removal or specific complex isolation.[20] Covalent adducts of small ligands and enzymes are even simpler to probe since, in the covalent adduct, the bound moiety acquires the diffusion characteristics of the enzyme that is expected to differ dramatically from the free ligand in terms of the diffusion coefficient. Thus, complexed systems that contain covalent conjugates, reversible adducts and free ligands can easily be analyzed and assigned using diffusion NMR. This technique is of particular importance in multispecies mixtures with overlapping chemical shifts. In addition, ^{31}P diffusion NMR is a sensitive tool for studying ligand−enzyme interactions because of the very large differences in the molecular weights that result in substantial differences in the expected diffusion coefficients of these molecular species. Diffusion NMR provides complementary information that may not be available from chemical shift measurements.

The main drawback of the method, especially if one uses the ^{31}P NMR signal to measure diffusion, is the relative long acquisition time. This arises from the relatively low γ value of the ^{31}P nucleus, its relatively short T_2, and the low concentration of the phosphorus nucleus in the sample. In diffusion experiments one needs to annihilate the signal; therefore, the starting point should be spectra with reasonable SNR. Since several

(19) Derrik, T. S.; Licas, L. H.; Dimicoli, J.-L.; Larive, C. K. *Magn. Reson. Chem*. **2002**, *40*, S98−S115.

(20) (a) Lin, M.; Shapiro, M. J.; Wareing, J. R. *J. Org. Chem.* **1997**, *62*, 8930−8931. (b) Bleicher, K.; Lin, M.; Shapiro, M. J.; Wareing, J. R. *J. Org. Chem.* **1998**, *63*, 8486-8490. (c) Anderson, R. C.; Lin, M.; Shapiro, M. J. *J. Comb. Chem.* **1999**, *1*, 69−72. (d) Lin, M.; Shapiro, M. J.; Wareing, J. R. *J. Am. Chem. Soc.* **1997**, *119*, 5249−5250; e) Chen, A.; Shapiro, M. J. *Anal. Chem*. **1999**, *71*, 669A−675A.

such points need to be collected, these ^{31}P diffusion NMR experiments require a long acquisition time. In fact, although we used the STE diffusion sequence, which is more suitable for nuclei that have a short T_2, the diffusion experiment shown in Figure 4, for example, was collected during 72 h in a 4.0 mM solution of Cht (a relatively high enzyme concentration) on our 500 MHz spectrometer. It should be noted also that such concentrations may well affect the solution viscosity and may also result in some self-aggregation of the measured species.[18]

The information gained from diffusion NMR can thus be used to challenge the assignments deduced from chemical shift arguments and may assist in the determination of the actual species that prevail following enzyme-ligand interactions. In addition, the method allows the determination of diffusion coefficient of certain enzymes for systems in which the ^1H NMR spectra are too broad, using standard NMR equipment.

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