trans-3-Hydroxy-6-phenyl-1-(trimethylsiloxy)-1-cyclohexene (**45**): IR (CCl_4) 850, 910, 1260, 1605, 1655, 2950, 3045, 3600 cm^{-1}; ^1H NMR ($CDCl_3$, 360 MHz) 0.11 (9 H, s), 1.3 (s, 1 H, OH), 1.51-1.58 (1 H, m), 1.61-1.72 (1 H, m), 1.80-1.88 (1 H, m), 2.19-2.30 (1 H, m), 3.416 (1 H, dd, J = 5.37, 5.61 Hz), 4.454 (1 H, dt, J = 3.9, 4.64 Hz), 5.212 (d, J = 3.9 Hz); ^{13}C NMR ($CDCl_3$) 0.073, 27.810, 28.568, 45.687, 65.840, 108.528, 126.081, 127.056, 128.085, 128.627, 154.522; mass spectrum, m/z 244 (84%, M - H_2O), 229 (10), 211 (11), 167 (5), 153 (36), 73 (100).

6-Phenyl-2-cyclohexenone[41] (**46**). HCl (2%) was used to hydrolyze a sample of crude alcohol **45** to enone **46**, purified by column chromatography (silica gel, petroleum ether:ether, 3:1): IR (CCl_4) 1240, 1680, 2925, 3025 cm^{-1}; ^1H NMR (CCl_4, 60 MHz) 1.9-2.4 (4 H, m), 3.42 (1 H, br t, J = 7 Hz), 5.95 (1 H, dt, J = 10, 1.5 Hz), 6.7-7.3 (6 H, m); mass spectrum, m/z 172 (67, M), 104 (100%), 68 (95, M - 104).

3-Methyl-6-phenyl-2-cyclohexenone[42] (**48**): IR (CCl_4) 1675, 2935, 3035, 3070 cm^{-1}; ^1H NMR (CCl_4, 60 MHz) 1.70-2.30 (7 H, m), 3.00-3.45 (1 H, m), 5.63-5.80 (1 H, m), 6.80-7.35 (5 H, m); mass spectrum, m/z 186 (70%, M), 141 (2), 128 (3), 115 (8), 104 (33), 82 (100), 78 (11).

trans-5-Hydroxy-1-(trimethylsiloxy)-6-vinyl-1-cyclohexene (**49**). 1,2-Adduct **49** was obtained by reaction of 184 mg (1 mmol) of epoxy enol ether **11** with 5 equiv of (CH_2=CHCuCN)MgBr as 180 mg (85%) of a lightly colored oil. Compound **49** could also be obtained by reaction of 184 mg (1 mmol) of **11** with 3 equiv of (CH_2=CHCuCN)Li, yielding 170 mg (80%) of crude adduct, which could be purified by column chromatography on silica gel (hexane:ether, 3:1) but soon decomposed upon standing neat at room temperature. **49**: IR (CCl_4) 855, 1255, 1655, 2965, 3620 cm^{-1}; ^1H NMR ($CDCl_3$, 360 MHz) 0.105 (9 H, s), 1.462-1.561 (1 H, m), 1.764-1.840 (1 H, m), 2.009-2.089 (2 H, m), 2.11 (br s, OH), 2.640 (1 H, br t, J = 6.9 Hz), 3.622 (1 H, ddd, J = 6.2, 2.9 Hz), 4.832 (1 H, m), 5.097-5.155 (2 H, m), 5.535-5.635 (1 H, m); ^{13}C NMR ($CDCl_3$) 0.236, 20.172, 27.377, 53.813, 70.661, 103.545, 117.955, 137.836, 148.400; mass spectrum, m/z 226 (14, M), 168 (45), 153 (7), 143 (38), 73 (100).

3β-Hydroxy-3α-methyl-1-(trimethylsiloxy)-6α-vinyl-1-cyclohexene (**50**): IR (CCl_4) 855, 1255, 1655, 2965, 3620 cm^{-1}; ^1H NMR ($CDCl_3$, 360 MHz) 0.132 (9 H, s), 1.237 (3 H, s), 1.51-1.65 (2 H, m), 1.82 (br, OH), 1.889-1.985 (2 H, m), 2.649 (br, $W_{1/2}$ = 14 Hz), 4.810 (1 H, s), 4.960-5.009 (2 H, m), 5.641-5.736 (1 H, m); ^{13}C NMR ($CDCl_3$) 0.236, 25.101, 30.240, 33.932, 42.979, 69.469, 111.887, 115.192, 138.107, 153.547; mass spectrum, m/z 226 (14, M), 168 (45), 153 (7), 143 (38), 73 (100).

3-Methyl-6-vinyl-2-cyclohexenone (**51**). A sample of crude alcohol **50** was hydrolyzed with 2% HCl in the usual way, giving a nearly quantitative yield of enone **51**, which was purified by column chromatography on silica gel (AcOEt:hexane, 1:3) but quickly decomposed upon standing, even in solution. ^1H NMR (CCl_4, 60 MHz) 1.93 (3 H, br s), 1.6-2.4 (4 H, m), 2.6-3.0 (1 H, m), 4.8-5.2 (2 H, m), 5.7-6.3 (2 H, m); mass spectrum, m/z 136 (24, M), 121 (4), 108 (2), 82 (100), 54 (22).

5β-Hydroxy-4α-methyl-1-(trimethylsiloxy)-6α-vinyl-1-cyclohexene (**52a**). Epoxy enol ether **44** (198 mg, 1 mmol), containing ca. 40% of its C4 epimer, was treated with 3 equiv of (CH_2=CHCuCN)Li in the usual way, yielding 160 mg (70%) of a mixture of 1,2 and 1,4 adducts. Assignment of the signals in the 360-MHz ^1H NMR spectrum of the crude mixture was facilitated by hydrolyzing the mixture with 10% HCl (5 min, room temperature); from this new crude mixture, the only compound that could be completely separated (silica gel, petroleum ether:ether, 3:1) was the 1,2-adduct **52a**. Nevertheless, this allowed the assignment of the 360-MHz ^1H NMR spectrum of the original crude mixtures: the ratio of 1,4 to 1,2 addition was estimated to be 7:3. **52a**: IR (CCl_4) 1255, 1655, 2965, 3620 cm^{-1}; ^1H NMR ($CDCl_3$, 360 MHz) 0.121 (9 H, s), 1.044 (3 H, d, J = 6.12 Hz), 1.608-1.705 (1 H, m), 1.734-1.780 (1 H, ddd, J = 2.2, 5.8, 10.9 Hz), 2.040-2.111 (1 H, m), 2.675 (1 H, br t), 3.108 (1 H, dd, J = 9.77, 8.79 Hz), 4.813 (1 H, ddd, J = 5.61, 2.19, 1.95 Hz), 5.157-5.246 (2 H, m), 5.569-5.670 (1 H, m); ^{13}C NMR ($CDCl_3$) 0.182, 15.188, 30.627, 34.582, 55.005, 65.786, 103.653, 118.821, 138.432, 142.766; mass spectrum, m/z 226 (9, M), 208 (5), 193 (3), 168 (39), 156 (9), 75 (41), 73 (100).

Acknowledgment. This research was supported in part by a grant from the National Cancer Institute (CA 22237). We also wish to acknowledge NSF for providing departmental funds for the purchase of a JEOL-FX90Q and a Brüker 360-MHz NMR spectrometer.

Registry No. 11, 77326-17-1; **12**, 77326-15-9; **13**, 81360-46-5; **14**, 81360-47-6; **15**, 81422-57-3; **16**, 81360-48-7; **17**, 81360-49-8; **18**, 81360-50-1; **19**, 6610-21-5; **19** 2,4-dinitrophenylhydrazone, 52456-88-9; **20**, 81360-51-2; **21**, 38510-79-1; **22**, 81371-74-6; **23**, 15329-10-9; **24**, 81360-52-3; **25**, 49748-84-7; **26**, 81360-53-4; **26b**, 81360-54-5; *cis*-**27**, 22886-16-4; *trans*-**27**, 22886-15-3; **28**, 81360-55-6; **29**, 81360-56-7; **30**, 81360-57-8; **31**, 81360-58-9; **32**, 81360-59-0; **33**, 40790-56-5; **34**, 81360-60-3; **35**, 81360-61-4; **36**, 81360-62-5; **37**, 81360-63-6; **38**, 81360-64-7; **39**, 23438-77-9; **39** 2,4-dinitrophenylhydrazone, 81360-65-8; **40**, 81360-66-9; **41**, 81360-67-0; **42**, 1,2-adduct, 81360-68-1; **42**, 1,4-adduct, 81360-69-2; **43**, 81360-70-5; **44**, isomer 1, 71911-83-6; **44**, isomer 2, 81422-58-4; **45**, 81360-71-6; **46**, 36702-38-2; **48**, 6286-53-9; **49**, 81360-72-7; **50**, 81360-73-8; **51**, 77326-22-8; **52a**, 81360-74-9; **52b**, 81360-75-0; **53**, 81360-76-1; **54**, 81422-59-5; **64b**, 81360-77-2; **62**, 81360-78-3; **63**, 81360-79-4; **64**, 81360-80-7; **65**, 40122-96-1; 3,4-dimethyl-2-cyclohexenone, 10463-42-0; 3,4-dimethyl-2-cyclohexenol, 81360-81-8; 2,3-epoxy-3,4-dimethylcyclohexanol, 81360-82-9; 3-methyl-2,3-epoxycyclohexanone, 21889-89-4; 2,3-epoxy-2-methylcyclohexanone, 21889-75-8; (MeCuCN)Li, 41753-78-0; (*t*-BuCuCN)Li, 78856-98-1; (*n*-BuCuCN)Li, 41742-63-6; (PhCuCN)MgBr, 81360-45-4; (C_2H_3CuCN)MgBr, 81371-72-4; (C_2H_3CuCN)Li, 77043-46-0; diethyl chlorophosphate, 814-49-3; 3-methyl-2-cyclohexenone, 1193-18-6.

Supplementary Material Available: A complete NMR analysis (360 MHz ^1H and ^{13}C) of the stereochemistry of the adducts from cuprate reactions and chemical evidence for stereochemistry and experimental section for several enones and epoxides (19 pages). Ordering information is given on any current masthead page.

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Motion at the Active Site of Tosylchymotrypsin

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Abstract: Tosylchymotrypsin, deuterium or carbon-13 labeled in the tosyl group, has been prepared and examined by deuterium or carbon NMR spectroscopy. Analysis of spectral line widths indicates that rotation of both the methyl group and the aromatic ring of this moiety is rapid, although aromatic ring motion may be slowed slightly in the associated protein. The chemical shift of the methyl carbon is essentially invariant to sample pH or to denaturation of the protein. When considered in light of the structure of the crystalline protein and the effect of solvents on carbon chemical shifts, our collective observations suggest that the local structure of the active site in tosylchymotrypsin in solution is rather "loose", such that the tosyl group rotates freely and resides in an environment that is solvent rich.

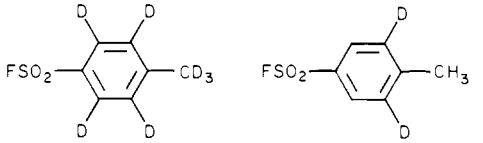
Covalent, substate-derived intermediates are often formed during enzyme catalysis. The presence of the covalently linked group which originated in the substate can stabilize the protein against structural changes induced by pH variation[1] or addition

0002-7863/82/1504-3172$01.25/0

of denaturing agents such as urea or detergents.[2] Such stabilization is in accord with the notion that, after recognition of a substrate, the enzyme structure around the substrate will tighten, so as to favor the alignment of catalytically important functional groups.

Tosylchymotrypsin is a stable analogue of the acyl-enzyme intermediate(s) formed during the action of the protease α-chymotrypsin. Produced by the action of tosyl fluoride on the enzyme, this protein has a single toluenesulfonyl group attached to serine-195, the group which becomes acylated during the hydrolysis of carboxylate derivatives. Crystallographic studies show that in the solid state the tosyl group is sandwiched between cysteine-191 and aspartate-194 on one side of the aromatic ring and a stretch of polypeptide between positions 213 and 217 on the other.[3] The cavity on the enzyme surface identified as a substrate recognition site is not completely filled by the tosyl group, and two water molecules are observed at the back of this pocket. Space-filling models suggest that the tosyl group is within van der Waals contact of the groups mentioned above,[4] and we anticipate that, while rotation of the methyl group of the tosyl derivative would likely be rapid in this structure, rotation of the aromatic ring may well be substantially hindered.

As a result of NMR studies of the structure and motion of a fluorinated analogue of tosylchymotrypsin,[5,6] we became interested in knowing more about the dynamics of motion of the tosyl group of tosylchymotrypsin in solution. Since deuterium nuclear relaxation is dominated by a single well-defined process, the quadrupolar mechanism,[7] modified enzymes were prepared with deuterated tosyl fluorides I and II and examined by deuterium



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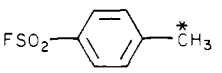

III

NMR spectroscopy. Carbon-13 relaxes by different mechanisms and carbon shifts are sensitive to molecular environment. For illumination of some of our conclusions, tosylchymotrypsin was therefore also prepared by using tosyl fluoride III, wherein the methyl carbon has been enriched to 90% carbon-13. The results described here suggest that in solution the tosyl group of tosylchymotrypsin is highly mobile, with rapid rotation of both the aromatic ring and the methyl group taking place in an environment that has the polarity of solvent water.

Experimental Section

Materials. Toluene-d_8 was purchased from Stohler Isotope Chemicals and was at least 99% deuterated. Toluene-*methyl*-^{13}C was obtained from KOR Isotopes, Cambridge, MA. This material was a pink liquid but had an NMR spectrum consistent with 90% carbon-13 enrichment at the methyl carbon.

***p*-Toluene-d_7-sulfonyl chloride** was prepared by a procedure based on published patents[7] and designed to optimize the yield of the para isomer. To 1 mL (15 mmol) of chlorosulfonic acid (Mallinckrodt) contained in a three-neck flask, equipped with a magnetic stirrer, condenser, drying tube, and dropping funnel, was added 0.8 g (15 mmol) of ammonium chloride. The mixture was heated to 80 °C and 1 g (10.8 mmol) of toluene-d_7 added dropwise over the course of 0.5 h. The remaining toluene in the addition funnel was washed into the reaction mixture with a few drops of chloroform. After addition was complete, the mixture was cooled to 35 °C and another milliliter of chlorosulfonic acid was added. Stirring continued for 1 h at the lower temperature, whereupon the mixture was poured onto 20 mL of ice and extracted with CH_2Cl_2 (3 × 10 mL). The extracts were washed with 10% $NaHCO_3$ solution (1 ×) and water (1 ×) and then dried over $MgSO_4$. Removal of the solvent in vacuo afforded about 1.8 g of product, which contained approximately 10% of *o*-toluenesulfonyl chloride. Recrystallization of the crude products from petroleum ether (bp 30–60 °C) produced about 0.8 g of the para isomer (~40%), mp 69–70 °C (lit.[8] mp 71–72 °C), contaminated with less than 1% of the ortho isomer. NMR spectroscopy with a known amount of CH_2Cl_2 as an internal intensity standard showed that about 6% of the deuterons at the ring position ortho to the CD_3 group were lost during this step.

***p*-Toluene-d_7-sulfonyl fluoride** was prepared by treating the chloride with 4 equiv of KF in 50% (v/v) acetone/water.[9] After sublimation, the tosyl fluoride was obtained in 85–95% yield, mp 43 °C (lit.[8] mp 43–44 °C). Infrared spectroscopy and silver nitrate tests revealed no detectable chloride.

Attempts to prepare tosyl fluoride by fluorosulfonation of toluene produced a mixture typically containing 20% of the ortho isomer. Neither fractional distillation nor recrystallization resulted in an acceptable purification of this product.

p-Toluene-*methyl*-^{13}C-sulfonyl chloride and *p*-toluene-*methyl*-^{13}C-sulfonyl fluoride (III) were prepared by the same method with toluene-*methyl*-^{13}C.

3,5-Dideuterio-4-methylbenzenesulfonyl fluoride (II, tosyl-d_2 fluoride) was prepared by starting with *p*-nitrotoluene-d_2.[10] Reduction of this material to *p*-toluidine-d_2 followed the procedure of Jones;[11] the toluidine was converted to tosyl-d_2 chloride by diazotization at room temperature and pouring into glacial acetic acid saturated with SO_2 and containing $CuCl{\cdot}2H_2O$.[12] Temperature control in the second state was important, and best yields (~55%) were obtained when the reaction mixture was maintained at 47 °C. Conversion to tosyl-d_2 fluoride followed the fluoride-exchange procedure described above. Mass spectroscopy and NMR spectroscopy showed that the product was about 90% deuterated at the ring positions ortho to the methyl group.

***p*-Toluenesulfonyl fluoride** (unlabeled) was purchased from Aldrich Chemical Co.

3-(Dimethylamino)propyl tosylate hydrochloride was prepared by mixing 1.9 g (10 mmol) of tosyl chloride, 5 mL of $CHCl_3$, and 1 g (10 mmol) of 3-(dimethylamino)propanol (Aldrich) at 0 °C. After the mixture was stirred overnight, the product was removed by filtration, washed with $CHCl_3$, and dried in vacuo. The proton and carbon NMR spectra of the product were consistent with the expected structure.

α-Chymotrypsin (3× recrystallized) was obtained from Miles Laboratories (lot no. 7018) and was used without purification.

Tosylchymotrypsin was prepared as described by Sigler et al.[9] Activity of the modified enzyme was much less than 1% of that of the native enzyme when determined by hydrolysis of *p*-nitrophenyl acetate or *N*-glutarylphenylalanine *p*-nitroanilide.[18] The reaction mixtures were concentrated by ultrafiltration (Amicon UM-10 membranes) and then lyophilized.

Isoelectric focusing of both native and serine-modified enzymes generally show the presence of several contaminants.[18] In one experiment, native enzyme was chromatographed on CM-32 (carboxymethyl)cellulose at pH 7 with 0.1 M KCl as eluent, after an initial forerun with 0.1 M KCl as an eluent. The main fraction was collected directly as it eluted from the column into a vessel containing tosyl-d_2 fluoride reaction mixture. As discussed below, this procedure served to remove an NMR-detectable impurity.

Procedures. Protein concentrations were determined spectrophotometrically at 280 nm with a molar extinction coefficient of 5×10^4 m^{-1} cm^{-1}.[13]

Deuterium NMR spectroscopy was carried out at 76.77 MHz on the Bruker WM-500 instrument at the Southern California Regional NMR facility, at 30.8 MHz on a Nicolet NT-200 at Nicolet Magnetics Corp., and at 23.1 MHz on a Nicolet NT-150 operated by the Colorado State University Regional NMR Center. Samples for deuterium spectroscopy were 1.0–1.2 mM in protein and contained 0.1 M KCl; they were pre-

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pared by using deuterium-depleted water (Aldrich). The sample pH was adjusted to the stated values by addition of small amounts of concentrated HCl or NaOH made up in normal water, and the sample pH values were those observed with a Radiometer PHM52 meter. Sample temperature was not carefully controlled in the deuterium experiments but was approximately 24 °C in each case.

For estimation of the deuterium chemical shifts, the proton (^{1}H) shift of the water signal in each sample was measured relative to the signal from an external capillary of tetramethylsilane, which was taken as 0.0 ppm. It was assumed that the deuterium shift of the residual HOD would be the same, and deuterium shifts were referenced to the HOD signal in each spectrum.

Carbon spectroscopy at 25.4 MHz was carried out on a Varian XL-100 spectrometer equipped with accessories for multinuclei examination that were based on the approach of Traficante et al.[14] Sample temperatures were controlled at 25 ± 1 °C by the Varian controller. Carbon spectra at 50.3 MHz were obtained by using 12-mm diameter samples on a Nicolet NT-200 spectrometer maintained at the University of California, Davis campus. Dielectric heating of the samples by the proton decoupler on the latter instrument was a problem,[15] and while the variable temperature controller was set for 15 °C, it may be that the temperature within the sample was slightly higher than this. The number of data points representing the accumulated fid's was sufficient that the digital resolution of the transformed spectra was at least 0.03 ppm in all cases. A capillary tube of spectrograde dioxane was used to provide a reference signal; the dioxane singlet was assigned a chemical shift of 67.39 ppm downfield from tetramethylsilane.[16] The resonance for the enriched methyl group was located by difference spectroscopy; spectra of tosylchymotrypsin samples prepared in the same way as the tosylchymotrypsin (methyl-^{13}C) samples were collected under conditions identical with those used for the labeled materials. The singlet for the labeled carbon was clearly resolved in this manner, but some peak broadening and uncertainty in peak position inevitably result from this procedure. We estimate that the protein chemical shifts given are reliable to ±0.06 ppm while the peak widths are likely uncertain by about ±15%.

Samples for carbon NMR spectroscopy were prepared by adding the appropriate amount of solid KCl to enzyme solutions in which at least 50% of the solvent protons had been replaced by deuterium. The protein concentration in the samples was 1.5 mM while the concentration of KCl was 0.1 M. The apparent sample pH values reported are those indicated by the Radiometer PHM52 meter; no corrections for the deuterium content of the solvent were made. Sample pH was adjusted by addition of small amounts of KOH in D_2O or HCl in D_2O solution.

A Varian CFT-20 spectrometer was used for some of the solvent effect studies. Samples temperatures were 33 ± 1 °C but were not controlled, and the digital resolution was 0.05 ppm in the transformed spectra. A capillary of dioxane was again used to provide a reference signal; bulk susceptibility corrections were applied to the data by using the magnetic susceptibilities given by Emsley, Feeney, and Sutcliffe.[17]

Results

Deuterium NMR Spectroscopy. The samples for ^{2}H NMR spectroscopy were made up in deuterium-depleted water. Even so, the dominant feature of each deuterium spectrum was a sharp line at the HOD chemical shift corresponding to residual deuterium in the solvent. In addition to this resonance, the ^{2}H NMR spectra of specifically deuterated tosylchymotrypsins prepared with sulfonyl fluorides I and II consisted of broad lines with corresponding sharp lines on top of them. Figure 1 presents some typical observations when the deuterium observation frequency was 76.77 MHz. For determination of the line width of each resonance, the corresponding chemical shift, and the relative amount of the sharp and broad components, the experimental spectra were fit to a sum of Lorentzian curves by trial and error; the smooth curves in Figure 1 are simulations obtained in this way for tosyl-d_7-chymotrypsin (trace C) and tosyl-d_2-chymotrypsin.

Trace A of Figure 1 is the ^{2}H spectrum of tosylchymotrypsin of normal isotopic composition. Although twice as many free induction decays were accumulated to obtain this spectrum as were used for the other curves shown in Figure 1, there are no signals

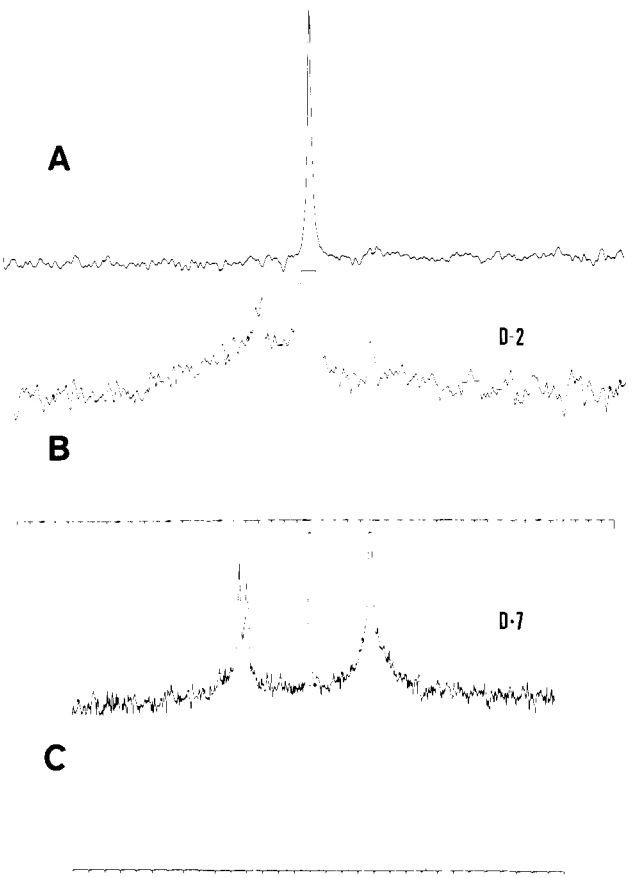

Figure 1. Deuterium NMR spectra obtained with an operating frequency of 76.77 MHz. (A) Tosylchymotrypsin of normal isotopic composition (1.0 mM) in deuterium-depleted water with 0.1 M KCl, adjusted to pH 7.1. The plot scale is 50 Hz/division; 26000 scans were collected for this spectrum. (B) Tosyl-d_2-chymotrypsin (1.0 mM), 0.1 M KCl at pH 5.3 (9000 scans). This sample contained 0.033 mM CD_3COO^- and the inactive protein was obtained by capturing native enzyme as it eluted from a CM-32 column with tosyl-d_2 fluoride. (C) Tosyl-d_7-chymotrypsin (1.2 mM), 0.05 M KCl at pH 6.2 (2000 scans). The smooth curves in each case are those calculated by summing Lorentzian curves at the position, amplitude, and width indicated in Table I.

observed for the deuterium nuclei that appear at natural abundance in the protein. Thus, the background signals for traces B and C are negligible.

In some experiments a small, known amount of sodium deuterioacetate (CD_3COONa) was included in the solution. This provided an intensity reference signal which was included when the simulated curves were prepared (trace B, Figure 1). After correction for signal losses during the exciting rf pulse and the delay before data accumulation, it was determined that at least 95% of the possible deuterium signal intensity in these samples was represented by the line shapes observed. That is, there is very little if any signal intensity appearing in lines broader than those indicated by the curve-fitting procedure.

Table I summarizes results obtained in fitting ^{2}H NMR spectra of both the tosyl-d_2- and the tosyl-d_7-enzymes. The amount of the material(s) giving rise to the sharp lines in crude inactivated protein was 25–35%; the contribution of these signals to the spectra could be reduced by ultrafiltration of the samples through a UM-10 membrane (Amicon) and minimized by treating the native enzyme with deuterated tosyl fluoride as it emerged from chromatography on a (carboxymethyl)cellulose column. These observations are similar to those made by fluorine NMR spectroscopy with *p*-(trifluoromethyl)benzenesulfonylchymotrypsin,[18] the sharp resonances observed with that system and the present one likely arise from autolyzed or denatured enzyme or enzyme fragments.

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Table I. Analysis of Deuterium NMR Spectra[a]

		broad component				sharp component					
		aromatic		methyl		aromatic		methyl			
system	pH	shift, ppm	$w^{1/2}$, Hz	shift ppm	$w^{1/2}$, Hz	shift ppm		$w^{1/2}$, Hz	shift, ppm	$w^{1/2}$, Hz	%[b]
d_7	4.4	8.0	80	2.7	40		c		c	c	94[d]
d_7	6.1	8.3	320	2.8	170	8.07	7.73	15	2.70	14	66
d_7	6.2	8.1	295	2.6	125	8.11	7.78	9	2.69	1	76
d_2	5.3	7.9	370				7.87	12			95[e]
d_2	6.9	c	295				d	7			85[d]
d_2[f]	7.0	c	416				d	5			90

[a] Samples were 1.0-1.2 mM protein. Sample temperature was approximately 24 °C. Unless otherwise noted the operating frequency was 76.77 MHz. [b] % of total signal intensity due to broad signal component. [c] Not reliably determined. [d] Protein solution was ultrafiltered to reduce impurities, giving sharp lines. [e] Modified protein prepared by capturing column effluent as described in Experimental Section. [f] At 30.8 MHz.

Table II. Methyl Carbon-13 Chemical Shifts of Tosylchymotrypsin

sample	rf, MHz	temp, °C	δ(obsd)	δ(cor)[a]	$\Delta w_{1/2}$, Hz
		Native Protein			
pH 3	50.3	~15	20.97	21.30	5
pH 7	50.3	~15	21.09	21.42	30
pH 7	25.4	25	21.57	21.57	32
pH 10	50.3	~15	21.01	21.34	29
		Denaturated (8 M Urea) pH 7			
	50.3	~15	21.04	21.37	25

[a] Chemical shifts were corrected to 25 °C, assuming a temperature coefficient of 0.033 ppm/deg for the shift relative to external dioxane (67.39 ppm).

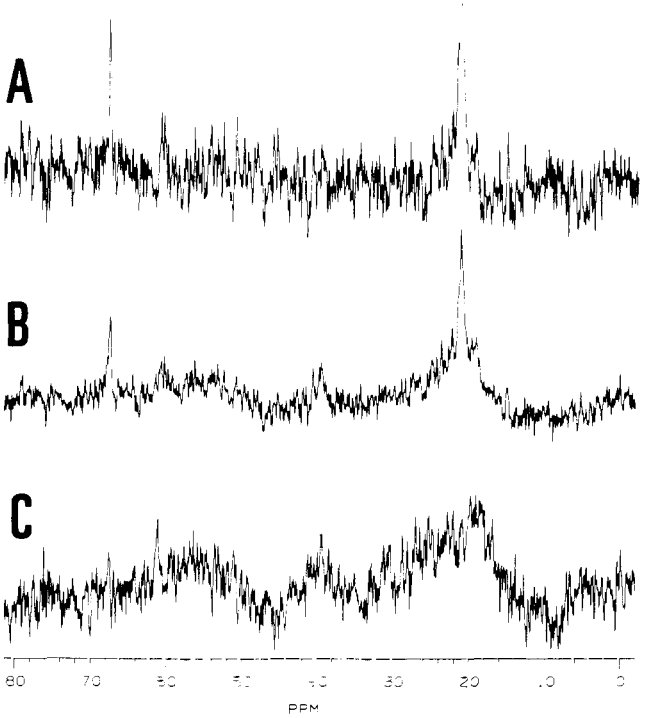


Figure 2. Carbon-13 spectra of native tosylchymotrypsin at pH 7, ~15 °C, 50.3 MHz, with complete proton decoupling. Trace A is the difference spectrum formed from the spectra of tosyl-methyl-^{13}C-chymotrypsin (trace B) and tosyl-methyl-^{12}C-chymotrypsin (trace C). The downfield resonance at 67.39 ppm arises from the dioxane reference.

Native tosylchymotrypsin is presumably represented by the broad resonances.

Carbon-13 NMR Spectroscopy. Figure 2 indicates the quality of the carbon-13 NMR data that could be obtained with ca. 1.5 mM solutions of tosylchymotrypsin that had been prepared with sulfonyl fluoride III; the resonance for the enriched carbon is clear in trace C but is made more obivous in the difference spectrum (trace A). The chemical shift and the approximate line width at half-peak height of the enriched methyl were determined by the difference technique at two resonance frequencies and at several values of sample pH and temperature. These data are collected in Table II. Studies with model compounds as well as the protein indicated that chemical shifts measured relative to a capillary of neat dioxane had a temperature variation of approximately 0.033 ppm/deg, and shift data corrected to 25 °C with this coefficient are given in the table.

Spectra were also obtained with tosylchymotrypsin dissolved in 8 M urea. These conditions denature the native enzyme[19] and,

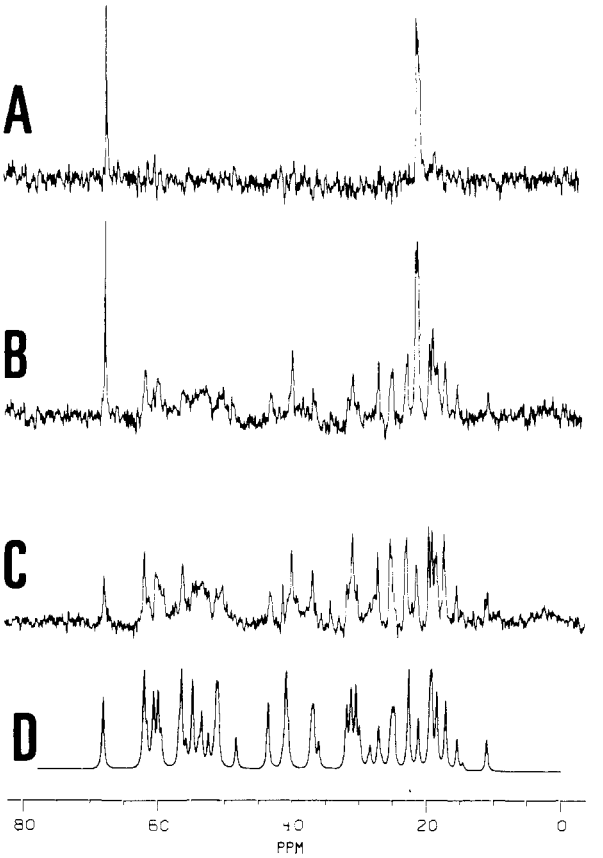


Figure 3. Carbon-13 spectra of tosylchymotrypsin in 8 M urea at pH 7, ~15 °C, 50.3 MHz, with complete proton decoupling. Trace A is the difference between spectrum obtained when spectra of the methyl-^{13}C enzyme (trace B) and the methyl-12C enzyme (trace C) are substracted. Trace D is a calculated spectrum for the denatured enzyme obtained by using the known amino acid composition of the protein and the peptide chemical shifts given in ref 20. The line width at half-height for a single resonance in the simulation is 0.35 ppm. The downfield peak at 67.39 ppm is due to the external dioxane reference.

Table III. Solvent Effects on Model Compounds[a]

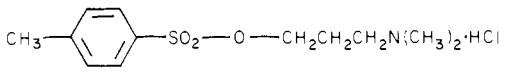
solvent	tosyl-ester (IV) methyl,[b] δ	propionitrile (V) methyl,[c] δ
deuterium oxide	21.56	10.46
acetone-d_6	21.75[d]	10.70
dimethyl-d_6 sulfoxide	22.17	11.57
chloroform-d	22.61	11.39
8 M urea in D_2O	21.78	10.59

[a] Solute was 0.8 M in the solvent indicated. Bulk susceptibility corrections were applied except for 8 M urea in D_2O. Chemical shifts are relative to the signal from an external capillary of dioxane which was set at 67.39 ppm. [b] At 25 °C. [c] At 33 °C, resonances were assigned by off-resonance proton decoupling. [d] Solvent was 90% acetone-d_6/10% D_2O in this case.

given the considerable sharpening that is observed (Figure 3), must have the same effect on the tosylated protein. Difference methods unambiguously located the resonance of the enriched methyl carbon, and its position as given in Table II is within experimental error of that observed for the native protein.

A simulation of the spectrum expected for the denatured enzyme carried out by using the chemical shifts of the various amino acids given by Howarth and Lilley[20] and the relative amounts of each amino acid in α-chymotrypsin is seen in Figure 2, trace D. The agreement between the simulation and the experimental spectrum of the enzyme in 8 M urea is reasonably good and confirms the conclusion that the protein is denatured under these conditions.

Medium Effects on Model Compounds. In order to explore the range of carbon chemical shift effects to be expected upon change of medium, we determined the methyl carbon chemical shifts for two compounds in a series of solvents. To examine solvent effects on the shifts of a tosyl ester, we settled upon compound IV as a model for the tosylated serine residue of the enzyme derivative; this material was chosen because it is soluble in water and several organic solvents. Chemical shift data for IV are recorded in Table III. Solvent effects on the methyl chemical shifts in a simpler system, propionitrile (V), were also determined and are summa-



IV

rized in Table III. The magnitude of the chemical shift changes are similar for both compounds, indicating that the charge on model IV does not substantially influence the way a given solvent perturbs the carbon chemical shift. Chemical shifts for IV were obtained at several concentrations in water and chloroform, and the shift at infinite dilution was obtained by extrapolation since it was suspected that IV might form micelles under the conditions of the experiments. The shifts at infinite dilution were less than 0.15 ppm different from the values given in Table III.

Experiments with these model systems suggest that transfer of a tosyl methyl group from water to a low dielectric constant, relatively nonpolar environment such as that provided by dimethyl sulfoxide or chloroform should produce a measurable downfield chemical shift effect at the carbon atom of the methyl group.

Analysis of Molecular Motion. Spin–spin relaxation of the deuterium nucleus is dominated by the quadrupolar mechanism, to the extent that dipolar contributions or the effects of possible paramagnetic impurities can be neglected. For quadrupoles with $I = 1$, the relaxation process is governed by a single exponential term and the line shapes are simple Lorentzian curves.[21] The width of these lines at half-height ($\omega_{1/2}$) is given by[22]

$$\omega_{1/2} = \frac{1}{160\pi} Q_D^2 (1 + \eta^2/3)[9J(0) + 15J(\omega) + 6J(2\omega)] \quad (1)$$

where Q_D is the quadrupolar coupling constant for the deuteron under consideration, η is an asymmetry parameter, and the spectral density functions (J) depend upon the details of molecular motion of the fragment holding the deuterium nucleus. We may take as Q_D for the aromatic and methyl deuterons of tosylchymotrypsin 193 and 165 kHz, respectively; these are the values found for benzene-d_6 and toluene-d_3.[23-25] The asymmetry parameter η likely does not exceed 0.06 and can be neglected for the present calculations.[26]

The simplest assumption to make is that the molecular framework bearing the deuterium nuclei tumbles isotropically such that the motion can be described by a single correlation time, τ_c. In this case, the form of the spectral density functions is

$$J(\omega) = \tau_c/[1 + (\omega\tau_c)^2] \quad (2)$$

When the experimental line widths (Table I) for deuterated tosylchymotrypsin are used, τ_c for the aromatic deuterons is estimated to be 5 ± 1 ns at pH 7 and about 0.5 ns at pH 4.

The correlation time for overall tumbling of monomeric chymotrypsin is within the range 11–16 ns, as deduced by a variety of methods.[27-30] Thus, the aromatic deuterons of deuterated tosylchymotrypsin enjoy freedom of motion beyond that of simple tumbling of the protein. That is, these nuclei cannot be rigidly locked in the protein structure.

At another level of approximation we may consider a model in which diffusive internal rotation of the tosyl aromatic ring and methyl group can take place such that a given carbon–deuterium bond is at an angle θ relative to the rotation axis; the rotation axis itself is assumed to be rigidly attached to the protein and thus tumbles with the characteristic correlation time (τ_c). The appropriate spectral densities then are[31]

$$J(\omega) = \frac{(3\cos^2\theta - 1)^2}{4} \frac{\tau_c}{1 + (\omega\tau_c)^2} + (3\sin^2\theta\cos^2\theta) \times \frac{\frac{6\tau_c\tau_i}{\tau_c + 6\tau_i}}{1 + \left(\frac{\omega\tau_c\tau_i}{\tau_c + 6\tau_i}\right)^2} + \frac{3\sin^4\theta}{4} \frac{\frac{3\tau_c\tau_i}{2\tau_c + 3\tau_i}}{1 + \left(\frac{3\omega\tau_c\tau_i}{2\tau_c + 3\tau_i}\right)^2} \quad (3)$$

where τ_i is a correlation time describing the internal rotation process. If we assume that rotation of the aromatic ring or methyl group is rapid ($\tau_c \gg \tau_i$), then eq 3 can be approximated by

$$J(\omega) = \frac{(3\cos^2\theta - 1)^2}{4} \frac{\tau_c}{1 + (\omega\tau_c)^2} \quad (4)$$

The deuterated methyl group of tosylchymotrypsin surely is in the rapid rotation limit, and calculation using the CD_3 line widths give $\tau_c \sim 10$ ns at pH 4 and $\tau_c \sim 35$ ns at pH 7, assuming $\theta = 70°$.

When the chemical shift tensor of toluene is used, calculations indicate that the chemical shift anisotropy contribution to the line width of the carbon-13-labeled methyl group is negligibly small.[32] When it is then assumed that the transverse relaxation of the methyl carbon is dominated by proton–carbon dipolar interactions and eq 3 for the spectral densities is used, the carbon line width

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observed at low pH is consistent with rapid rotation of the methyl group and τ_c in the range 10–15 ns.[33] At neutral pH, the line width observed indicates τ_c in the range 35–50 ns with rapid internal rotation. The correlation time (τ_c) for overall tumbling of the protein depends on its aggregation state; previous work has shown that the tosyl-enzyme associates nearly as well as the native enzyme at pH 5–7[34] while at pH < 4 association is much less.[34,35] The deuterium and carbon-13 relaxation data for the methyl group are thus parallel; at pH 4 the protein appears to be monomeric but at pH 7 tumbles more slowly, indicating a dimeric or oligomeric structure in solution.

With the values of τ_c defined by the methyl carbon and deuterium relaxation data, one can then inquire about what value of τ_i for rotation of the aromatic ring is consistent with the data. At pH 4 and pH 7, $\tau_i \sim 0.14$ and ~ 1.1 ns, respectively, give calculated line widths within experimental error of those observed. The correlation times at high pH give a calculated line width at 30.8 MHz in agreement with that observed experimentally.

Rather than diffusive rotation, the aromatic rings of a protein may also rotate by a "180°-flip" mechanism in which these rings appear to librate only slightly about their equilibrium orientation before flipping 180° in a large-scale conformational excursion.[36,37] With τ_f representing the lifetime of the aromatic ring in one orientation before it flips 180°, the spectral densities for this model of aromatic group motion in proteins are given by

$$J(\omega) = \left(\frac{3\sin^4\theta + (3\cos^2\theta - 1)^2}{4}\right)\frac{\tau_c}{1 + (\omega\tau_c)^2} + (3\sin^2\theta\cos^2\theta)\frac{\frac{\tau_c\tau_f}{\tau_c + \tau_f}}{1 + \left(\frac{\omega\tau_c\tau_f}{\tau_c + \tau_f}\right)^2} \quad (5)$$

where θ and τ_c are the same as defined previously.[37] The effect of restricting the aromatic ring to this regime for rotation is to greatly reduce the line-narrowing effects of internal rotation. We were unable to find any values for τ_f that gave deuterium line widths even close to those observed if the values for τ_c defined by the analysis of the methyl group motion are used. The aromatic ring of the tosyl group thus appears not to interact strongly enough with other parts of the enzyme molecule that its rotational motion is restricted to 180° flips.

Discussion

Sulfonate esters of chymotrypsin are reasonably stable at neutral pH, although decomposition reactions become more rapid at either low or high acidities.[38,39] Phenylmethanesulfonylchymotrypsin has a half-life for hydrolytic degradation of about 6 h in 8 M urea at 10 °C. The time required for most of the spectra obtained in the present work did not exceed 4 h, and it was only with overnight accumulations ($\sim$10 h) for some of the carbon spectra that we noticed the appearance of a shoulder on the signal for the enriched carbon; this was assigned to toluenesulfonate cleaved from the enzyme. Thus, we believe that the spectral data given in Tables I and II represent results for systems in which the covalent bonds holding the tosyl group to the protein are still intact.

As indicated above, the observed deuterium line widths are narrower than expected for an aromatic ring tightly held in a protein structure the size of α-chymotrypsin. Although ambiguities may arise in specifying the quantitative aspects of the process,[40] it is clear that the tosyl group enjoys considerable freedom of molecular motion in tosylchymotrypsin. By use of the model chosen to describe this motion, the results suggest that at low pH the protein tumbles with a correlation time consistent with the monomeric form of the protein while the aromatic ring undergoes rapid rotation about its local symmetry axis. The results also indicate rapid rotation of the tosyl methyl group. In the solid state the tosyl group of this modified enzyme is essentially within van der Waals contact of the polypeptide chains that define the active-site "pocket". Should this structure be rigidly retained in solution, rotation of the aromatic ring could take place (at a considerable energy expense) only if the local protein structure is relaxed to permit turning the aromatic ring. Thus, our observations may suggest that the active-site structure of the enzyme becomes appreciably "looser" in solution such that the tosyl group retains the general orientation it has in the solid state relative to other features of the protein but is afforded more space for conformational motion. Alternatively, it may be possible for the tosyl group to equilibrate rapidly from its solid-state orientation to one in which it is oriented away from the protein toward soluion, where rotation of the aromatic ring can be very rapid.

Aromatic ring rotation appears to be appreciably slowed when the protein associates. Dimerization of the tosyl-enzyme in solution probably results in a structure similar to the dimer observed in the crystalline state.[3] In this structure the two (tosylated) active sites are face to face, and any propensity of the tosyl group to equilibrate to a conformation with the aromatic ring out of the specificity pocket would be reduced by interactions with the other protein molecule in the dimer.

Several effects could be experienced by the tosyl methyl carbon of tosylchymotrypsin that would result in enzyme-induced chemical shift effects being observed. These include (1) ring current effects from aromatic rings of the enzyme structure, (2) shielding changes due to bond angle or bond length distortions, and (3) medium (solvent) effects that arise when the methyl group is transferred from water to its protein environment.

Using the coordinates of crystalline tosylchymotrypsin[3] and the treatment of Mallion and Haigh,[41] we estimated the ring current effects of those aromatic residues closest to the tosyl methyl in the enzyme (tyrosine-228 and -146, tryptophan-172, -215, and -141, and phenylalanine-93). On this basis, all ring current shifts were computed to be quite small, and the cumulative effect of these aromatic amino acids should result in an upfield shift of less than 0.1 ppm.

Structural distortions large enough to cause chemical shielding changes require that there be appreciable van der Waals interaction between molecular fragments. Consideration of the crystal structure of tosylchymotrypsin suggests that the closest approach another atom makes to the tosyl methyl group is ~4 Å—a distance probably much too large to produce significant shielding changes by the Grant–Cheney mechanism.[42,43] Moreover, force-field calculations reinforce the intuitive conclusion that methyl groups are able to accomodate steric stress very easily by rotation and bending of bond angles,[43,44] and we conclude that shielding effects on the carbon chemical shifts of the tosyl methyl group due to steric compression are likely small.

Upon tosylation water molecules are displaced from the active site of chymotrypsin, although two waters remain at the back of the specificity pocket.[3] As indicated above, the methyl group of the tosyl moiety is surrounded largely by polypeptide. While it is likely not profitable to describe the environment of the methyl group in terms of the bulk properties of some organic solvent, it

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is clear that this environment is different from that which the methyl would experience in aqueous solution. Shift data for the methyl carbons of both the model compound IV and propionitrile (V) show that medium effects on the chemical shifts of methyl carbons can be appreciable. By the criterion of carbon chemical shifts, the environment of the tosyl methyl group of tosyl-chymotrypsin in solution appears to be in a highly polar one, similar in its properties to bulk water or possibly acetone.

Neither denaturation nor a possible conformational change that takes place at low pH[44] produces any appreciable change in the chemical shift of the tosyl methyl carbon. This situation could arise if the tosyl group does not reside full time in the specificity pocket but can be rapidly rotated so as to be exposed to solvent an appreciable fraction of the time. Alternatively, the active site found in the solid state could "expand" in solution such that the region around the methyl group is more hydrated than it is in the solid-state structure. Thus, the indications from the carbon chemical shift results are consonant with the high mobility of the tosyl group suggested by the deuterium line widths.

The time scale for motion of aromatic rings in proteins is quite broad.[36,37] For phenyl rings located deep inside a protein structure, rotation is usually slower than that observed for the tosyl ring of tosylchymotrypsin. However, groups on the surface of proteins tend to have high mobility, and in spite of the attractions of the active site, it is into this category that one must apparently place the tosyl group of the tosyl-enzyme.

Acknowledgment. We thank the National Institutes of Health for grants (CA-11220 and GM-25479) that supported this work and tender our appreciation to Dr. G. B. Matson of the University of California at Davis for his assistance with some of the experiments. The Southern California Regional NMR Facility is supported by NSF Grant CHE7916324A1; we thank Dr. W. R. Croasmun for his assistance with the deuterium spectroscopy. Some initial deuterium results were obtained at the Colorado State University Regional NMR Center, supported by NSF Grant CHE7818581, and we are indebted to Dr. Bruce Hawkins and Dr. James Frye for their help in this regard.

Registry No. I, 81255-46-1; II, 81255-47-2; III, 81255-48-3; IV, 39620-95-6; V, 107-12-0; *p*-toluene-d_7-sulfonyl chloride, 81255-49-4; *p*-toluene-*methyl*-^{13}C-sulfonyl chloride, 81255-50-7; chymotrypsin, 9004-07-3.

Resonance Raman and 500-MHz ^{1}H NMR Studies of Tyrosine Modification in Hen Egg White Lysozyme

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Abstract: Resonance Raman spectroscopy has been employed to monitor separately and simultaneously the behavior of two nitrated tyrosines in hen egg white lysozyme. The intensity of the NO_2 symmetric stretching mode was used to determine that the pK_as of the nitrated moieties were 6.76 ± 0.05 and 6.52 ± 0.05, respectively. The frequencies of this mode for each residue (1340 and 1328 cm^{-1}, respectively) were compared to model systems. The high-frequency band was characteristic of a nitrotyrosine residue in an exposed aqueous environment (residue 23), while the low-frequency band indicated a hydrophobic and hydrogen-bonded site for the second nitrotyrosine (residue 20 or 53). The results were consistent with 500-MHz ^{1}H NMR data and demonstrate the utility of the resonance Raman technique in discerning environmental changes around tyrosine residues.

Nitration of tyrosines on proteins is one of the more selective chemical modification techniques available. Since tyrosine is found at the catalytic centers of many enzymes, it is of importance to develop physical approaches to monitor the environment around this residue. While the nitrotyrosyl derivative that results from nitration is a UV-vis chromophore,[1] the relatively low information content inherent to this form of spectroscopy makes development of other techniques desirable. We have found that the intensity of the NO_2 symmetric stretching mode in the resonance Raman spectrum of 3-nitrotyrosine and of nitrotyrosyllysozyme can be employed to determine the pK_a of the nitrated moiety. In addition, the frequency of the vibration is strongly environment dependent.

The Raman experiment monitors separately and simultaneously the behavior of two nitrated tyrosines in lysozyme and leads to results consistent with ^{1}H NMR studies conducted at 500 MHz. Since the NO_2 group constitutes a relatively small perturbation in the protein structure, resonance Raman studies of nitrated enzymes offer a useful technique for monitoring local environments around tyrosine residues.

Experimental Section

Materials. Tetranitromethane, dried *Micrococcus luteus* cells, *N*-acetylglucosamine (NAG), and *N,N'*-diacetylchitobiose [$(NAG)_2$] were obtained from Sigma. Hen egg white lysozyme (E.C. 3.2.1.17), Lot 7138, No. 36-324, 6 times crystallized, was from Miles Laboratories, Ltd. Bio-Gel P-4 (200-400 mesh) was obtained from Bio-Rad Laboratories. All salts were analytical grade, and all solutions were prepared with doubly distilled water.

All pH measurements were made on a Radiometer Model 26 pH meter equipped with a glass combination microelectrode (GK 2321C). UV-vis data were obtained on a Cary 219 instrument.

Nitration of Lysozyme. A freshly prepared solution, containing 2 mg of tetranitromethane (10% solution in absolute ethanol), was added to a solution of lysozyme (58 mg/mL) in 0.05 M tris(hydroxymethyl)-aminomethane, 1 M NaCl, pH 8.0 buffer, so as to yield a 10-fold molar excess of tetranitromethane relative to enzyme concentration. The solution was stirred gently in the dark at 22 °C for 1 h. The reaction mixture was applied to a Bio-Gel P-4 column that had been equilibrated with doubly distilled water. The yellow nitroenzyme eluted as a single fraction in the void volume, desalted and well separated from the trinitroformate anion byproduct. The nitrotyrosylysozyme was lyophilized and stored at -20 °C until required.

Nitration of lysozyme in the presence of the inhibitors NAG and $(NAG)_2$ was carried out by using the above procedure after saturation of the enzyme with inhibitor [K_D for NAG = (4-6) × 10^{-2} M^2 and for $(NAG)_2$ = 6 × 10^{-4} M.[3]

The extent of nitration of the enzyme was determined spectrophotometrically. We employed ϵ_{381} (isosbestic) = 2200 M^{-1} cm^{-1}, ϵ_{428} = 4200 M^{-1}/cm^{-1} (nitrotyrosinate) for the quantitation of nitrotyrosines,[1c] and

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