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 tosylchymotrypsin 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

(1) (a) Riordan, J. F.; Sokolovsky, M.; Vallee, B. L. *Biochemistry* **1967**, *6*, 358-361. (b) Riordan, J. F. *Acc. Chem. Res.* **1971**, *4*, 353-360. (c) Muszyuka, G.; Riordan, J. F. *Biochemistry* **1976**, *15*, 46-51.

(2) Dahlquist, F. W.; Raftery, M. A. *Biochemistry* **1968**, *7*, 3269-3276.

(3) Sharon, N. *Proc. R. Soc. London, Ser. B.* **1967**, *167*, 402-415.

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Table I. Extent of Lysozyme Nitration for Various Experiments

experiment	[lysozyme], mM	[nitrotyrosyl-lysozyme], mM	[LiBr], M	pH	extent of nitration, NO_2/enzyme
LiBr denaturation, Raman	*a*	0.48	0	8.00	1.4
	a	0.48	3	8.00	1.4
	a	0.48	6	8.00	1.4
pH titration, Raman	*a*	0.83	*a*	5.25-8.11	1.5
nitration in presence of NAG, Raman	*a*	0.63	*a*	8.00	1.1
nitration in presence of $(NAG)_2$, Raman	*a*	0.63	*a*	8.00	1.4
NMR of lysozyme	3.9	*a*	*a*	4.88[b]	
NMR of nitrotyrosyllysozyme	*a*	5.1	*a*	4.56	1.3

[a] Sample contained 0.38 M $(NH_4)_2SO_4$ as an internal standard. [b] pH_{app}, measured at a glass electrode for the D_2O solution.

$\epsilon_{280}^{1\%} = 23.6$ for protein content.[4] The molar ratio of nitrotyrosines per enzyme was calculated by first correcting the ϵ_{280} for the maximum NO_2 absorbance as estimated from ϵ_{428}.[1c]

Enzyme Assay. The activity of lysozyme and chemically modified nitrotyrosyllysozyme was assayed according to the method of Shugar.[5] The method is based on the rate of decrease in apparent absorbance at 450 nm due to the lysis of *Micrococcus luteus* cells. The reaction mixture consisted of 2.9 mL of a stock solution of *M. luteus* cells (9 mg/30 mL) suspended in phosphate buffer (pH 7.0) and 0.1 mL of enzyme solution. The decrease in absorbance was recorded, and the change in absorbance per minute (ΔA_{450}/min) was determined over the initial linear portion of the curve. The activity was calculated as follows:

$$\frac{\text{units}}{\text{mg}} = \frac{\Delta A_{450}/\text{min} \times 10^3}{\text{mg of enzyme in reaction mixture}}$$

Raman Spectroscopy. Raman spectra were obtained with a Jarrell-Ash 3/4 meter double monochromator for dispersion, a Spectra-Physics Model 164 Ar^+ laser for excitation, and a Spex Industries DPC-2 photon counter for detection. The output from the DPC-2 was fed into a Spex Industries SCAMP computer which controlled the data acquisition and reduction. Typical spectral parameters were as follows: excitation frequency, 4880 Å; laser power, 600 mW; residence time, 1–2 s/cm^{-1}; number of scans, 10. Frequencies were calibrated by using atomic emission lines from a neon lamp. Frequency accuracy is estimated at 1–2 cm^{-1} for sharp features.

Samples for resonance Raman studies of 3-nitrotyrosine were prepared volumetrically from dilution of a stock solution with varying pH buffers. The 1280–1400 cm^{-1} and 875–975 cm^{-1} spectral regions were scanned. The area ratio of the NO_2 symmetric stretching mode at 1342 cm^{-1} to that of the ν_1 vibration (symmetric stretching mode) of the ClO_4^- internal standard at 935 cm^{-1} was plotted as a function of pH.

pH titrations of the enzyme were performed by adding equal volumes of a stock solution of nitrotyrosyllysozyme and $(NH_4)_2SO_4$ to 0.05 M citrate–phosphate buffer (pH 4.0) and to 0.05 M phosphate buffer (pH 8.0). The phosphate buffer solution was then titrated into the citrate–phosphate buffer solution. The regions 1280–1400 cm^{-1} and 950–1025 cm^{-1} were scanned at each pH. The area ratio of the NO_2 band contour to that of the symmetric stretching mode (983 cm^{-1}) of SO_4^{2-} (internal standard) was plotted against pH. Samples were contained in a rotating cell[6] with i.d. = 3.5 cm and height = 0.4 cm. Air or N_2 gas, cooled by passage through an ice–water bath, was blown over the cell to minimize local heating effects.

The individual contributions of the overlapped bands to the contour of the NO_2 stretching region of nitrotyrosyllysozyme were estimated as follows, by using a band decomposition procedure supplied by Spex Industries:

It was assumed that the observed contour was composed of two overlapped bands with frequencies of 1340 and 1328 cm^{-1}. A 50–50 Gaussian–Lorentzian mixture was found adequate to represent the peak shapes of the individual components. An initial guess for the relative intensities (with appropriate bandwidths for each band) led to a calculated contour, which was compared with that observed. From the difference between the calculated and observed contour, a new iteration cycle was generated. The calculation was halted when the difference between the current and prior iteration was 1% or less. In general, the average error between observed and calculated curves was less than 1%/data point. The areas of the individual components were then compared to that of the SO_4^{2-} standard at various pH values to generate titration curves for both the main band at 1340 cm^{-1} and the shoulder at 1328 cm^{-1}.

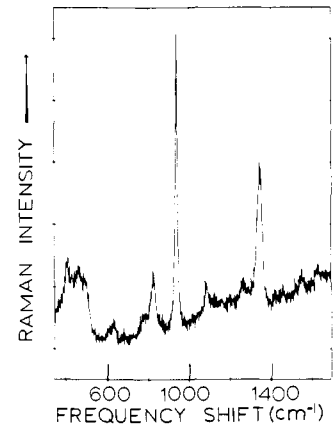

Figure 1. Resonance Raman spectrum of 3.0 mM 3-nitrotyrosine, pH 8.0. Excitation was 600 mW at 4880 Å. The NO_2 symmetric stretching mode appears at 1342 cm^{-1}. The intense feature at 935 cm^{-1} arises from the ν_1 stretching mode of ClO_4^-, which was added at a concentration of 1.5 M as an internal standard.

^{1}H Nuclear Magnetic Resonance. ^{1}H NMR spectra (54 °C) were obtained at 500 MHz in the Fourier transform mode at the Francis Bitter Magnet Laboratories at the Massachusetts Institute of Technology. A solution of lysozyme was prepared by dissolving 28 mg of protein in 0.5 mL of ^2H_2O (99.8%, Aldrich Chemical Co.) and adjusting the pH_{app} to 4.88. Nitrotyrosyllysozyme was prepared as described above with 1.3 molecules of nitrotyrosine per enzyme molecule according to spectrophotometric quantitation. A solution of nitrotyrosyllysozyme was prepared for ^{1}H NMR measurements by dissolving 37 mg of nitrotyrosyllysozyme in 0.5 mL of ^2H_2O and adjusting the pH_{app} to 4.56. The spectrum was resolution enhanced by convolution difference techniques.[7] An average of 1000 transients were collected for each spectrum.

Results

Extent of Nitration. The extent of nitration observed for the various samples examined in this study is shown in Table I. On the average, 1.4 ± 0.1 molecules of nitrotyrosine per enzyme molecule were found. This was the case even in the presence of the inhibitors NAG and $(NAG)_2$. Since the nitrating agent is the rather small NO_2^+,[8] the presence of the reversibly bound inhibitors does not appear to significantly alter the extent of nitration. Our results are also consistent with a recent report of Perkins et al.[9] which concluded that Gd(III) could bind simultaneously with *N*-acetylglucosamine. This again indicates that inhibitor binding does not preclude binding of other small species.

Nitration of less than one residue on lysozyme (ca. 0.7) did not significantly alter the activity. Upon nitration of 1.3 tyrosines, an ca. 50% loss in activity was observed.

Raman Spectroscopy. The resonance Raman spectrum of 3-nitrotyrosine as shown in Figure 1 exhibits a strong band at 1342 cm^{-1} assigned to the NO_2 symmetric stretching mode[10] along wiith

(4) Atassi, M. Z.; Habeeb, A. F. S. A. *Biochemistry* **1969**, *8*, 1385–1393.
(5) Shugar, D. *Biochim. Biophys. Acta* **1952**, *8*, 302–309.
(6) Kiefer, W.; Bernstein, H. J. *Appl. Spectrosc.* **1971**, *25*, 500–501.
(7) Campbell, I. D.; Dobson, C. M.; Williams, R. J. P.; Xavier, A. V. *J. Magn. Res.* **1973**, *11*, 172–181.
(8) Bruice, T. C.; Gregory, M. J.; Walters, S. L. *J. Am. Chem. Soc.* **1968**, *90*, 1612–1619.
(9) Perkins, S. J.; Johnson, L. W.; Phillips, D. C., Dwek, R. A. *Biochem. J.* **1981**, *193*, 573–578.

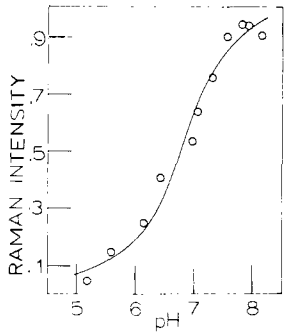

Figure 2. Resonance Raman pH titration curve for 3-nitrotyrosine. The ordinate scale is the normalized ratio of the area of the NO_2 symmetric stretching mode to the area of ν_1 for ClO_4^-. The theoretical curve (solid line) is drawn for a pK_a of 6.74. The circles are the experimental points. (The experimental points were obtained with 4579-Å excitation.)

several weaker features. The intensity of the spectrum as excited with Ar^+ laser lines at 4880 or 4579 Å is strongly pH dependent. The resonance Raman intensity of the anionic form is derived from vibronic coupling to the chromophore absorption band (428 nm), while the neutral molecules (chromophore λ_{max} = 360 nm) have minimal resonance Raman intensity with the concentrations and excitation wavelengths used in the current study. The resonance Raman spectra therefore report on the charged form of the molecule. The pH dependence of the symmetric NO_2 stretching mode intensity is shown in Figure 2. The non-resonance-enhanced ν_1 vibration of the ClO_4^- ion is used as an internal standard. The solid curve in Figure 2 corresponds to one theoretically calculated with a pK_a of 6.74 ± 0.05 and represents the best fit to the experimental data. This pK_a value is virtually identical with one previously observed by absorption spectroscopy.[1a] The feasibility of resonance Raman determination of chromophore pK_a is thus clearly shown from Figure 2.

As it was anticipated that the NO_2 frequency would respond to changes in the environment of the chromophore, nitrotyrosine was dissolved in various solvents and the resonance Raman spectrum obtained. The molecule was maintained in the ionic form by addition of LiOH to the hexane and chloroform solutions. Although nitrotyrosine was only slightly soluble in hexane and chloroform, resonance enhancement of the NO_2 mode was sufficient so that useful data could be obtained from dilute solutions. The symmetric stretching frequency of the NO_2 group was found at 1342 cm^{-1} in H_2O, 1339 cm^{-1} in ethanol, 1337 cm^{-1} in dioxane, 1336 cm^{-1} in hexane, and 1328 cm^{-1} in chloroform. Therefore, the NO_2 frequency responded to both alterations in solvent polarity and hydrogen-bonding ability, each characteristic inducing a reduction in frequency.[10] The largest shift observed (relative to aqueous solution) was in $CHCl_3$, where a 12-cm^{-1} lowering was found. In hexane, where no H bonding is expected, the shift was 6 cm^{-1}.

The resonance Raman spectrum of nitrotyrosyllysozyme unexpectedly showed two distinct features in the NO_2 stretching region (Figure 3). A main band at 1340 cm^{-1} was accompanied by a fairly well-defined shoulder at 1328 cm^{-1}. Resonance Raman spectra of protein nitrated in the presence of the inhibitors NAG or $(NAG)_2$ showed the same two features.

Resonance Raman spectra of nitrotyrosyllysozyme with progressively increasing concentrations of LiBr are shown in Figure 4. While spectra at 0 and 3 M LiBr are largely similar, denaturation of the enzyme with 6 M LiBr caused most of the 1328-cm^{-1} shoulder to merge into the main band (Figure 4); no frequency shift was observed for the latter. These observations, along with the NMR results discussed below, suggest that the two features in the resonance Raman spectrum of nitrotyrosyllysozyme arise from tyrosine residues in different environments. Denaturation of the enzyme renders the environment around each tyrosine equal. A merging of the two Raman features results, with the

(10) (a) Kumar, K.; Carey, P. R. *J. Chem. Phys.* **1975**, *63*, 3697–3707. (b) Gettins, P.; Dwek, R. A.; Perutz, R. N. *Biochem. J.* **1981**, *197*, 119–125.

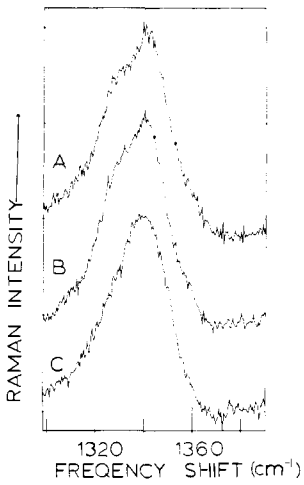

Figure 3. Resonance Raman spectra in the NO_2 symmetric stretching region of 0.48 mM nitrolysozyme in the presence of (A) no added LiBr, (B) 3 M added LiBr, and (C) 6 M added LiBr. Excitation was 600 mW of 4880 Å, pH 8.0. Upon denaturation with 6 M LiBr, the shoulder at 1328 cm^{-1} is seen to merge with the main peak at 1340 cm^{-1}.

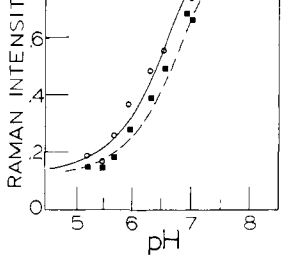

Figure 4. Resonance Raman pH titration curves for the main peak and shoulder of the NO_2 symmetric stretching modes of 0.48 mM nitrolysozyme. The open circles are the experimental area ratios for the 1328-cm^{-1} shoulder to the 983-cm^{-1} ν_1 band of the SO_4^{2-} ion used as an internal standard. The solid line is the theoretical curve calculated for a pK_a of 6.52. The closed squares are the experimental area ratios of the 1340-cm^{-1} band to the SO_4^{2-} ion ν_1 mode. The dashed line is the theoretical curve calculated for a pK_a of 6.76.

observed NO_2 symmetric stretching mode of the denatured enzyme appearing at a position (1340 cm^{-1}) identical within experimental error with that found for nitrotyrosine in aqueous medium.

The observed contour of the NO_2 region for nitrotyrosyllysozyme was decomposed as described in the Experimental Section. The pH dependence of the intensity of each component was obtained by plotting the area under each component relative to ν_1 of SO_4^{2-} (983 cm^{-1}) used as an internal standard. Data points were compared to theoretical pH curves, and the best fit was obtained by the method of least squares. A pK_a of 6.76 ± 0.05 was observed for the Raman feature at 1340 cm^{-1}, while the pK_a of the shoulder was found to be 6.52 ± 0.05. Both the pK_a and the frequency of the resonance Raman feature at 1340 cm^{-1} were similar to those in the 3-nitrotyrosine model. However, the frequency of the shoulder was shifted by 12 cm^{-1} while its pK_a was reduced by 0.2 or 0.3 unit compared with the model.

^1H NMR Studies on the Extent of Nitration. Since the three tyrosines of lysozyme had been observed and assigned before by Campbell, Dobson, and Williams,[11-14] it was of interest to de-

(11) Campbell, I. D.; Dobson, C. M.; Williams, R. J. P. *Proc. R. Soc. London Ser. B.* **1975**, *189*, 503–509.
(12) Campbell, I. D.; Dobson, C. M.; Williams, R. J. P. *Proc. R. Soc. London Ser. A*, **1975**, *345*, 23–40.
(13) Dobson, C. M. In "NMR in Biology"; Dwek, R. A., Campbell, I. D., Richards, R. E., Williams, R. J. P., Ed.; Academic Press: New York, 1977; pp 62–94.

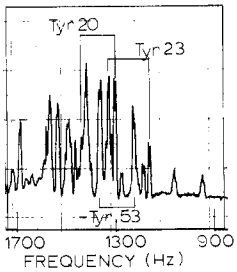

Figure 5. 500-MHz ^1H NMR spectrum of 3.9 mM native lysozyme at pH_{app} 4.88 in ^2H_2O recorded at 54 °C; 1000 transients were accumulated and Fourier transformed after application of the convolution difference parameters of 1, 4, and 0.95. The assignments of Tyr-23, Tyr-53, and Tyr-20 are taken from ref 14. The frequency is measured downfield from 4.6 ppm.

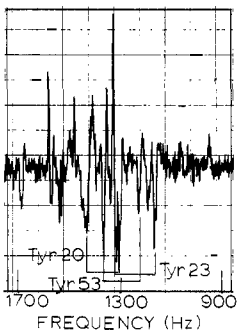

Figure 6. 500-MHz ^1H NMR spectrum of 3.9 mM native lysozyme at pH_{app} 4.88 in ^2H_2O recorded at 54 °C; 1000 transients were collected employing a Carr–Purcell sequence leading to multiplet selection. The choice of τ enabled inversion of doublets pertinent to tyrosines. Prior to Fourier transformation, convolution difference parameters of 1, 4, and 0.95 were applied. The frequency is measured downfield from 4.6 ppm.

termine which tyrosine was nitrated by tetranitromethane. Figure 5 presents the 500-MHz ^1H NMR spectrum at 54 °C for native lysozyme at approximately 3.9 mM concentration. The information content is enormous. Our interest is only in assigning the tyrosine resonances. For help in assigning these, further spectral simplification was employed. Perhaps the most useful of these is multiplet selection,[15] which allows separation of singlets and triplets from doublets. The tyrosine doublets are clearly seen inverted under these conditions, confirming the assignment in Figure 6. Upon nitration, the most upfield tyrosine appears to be most affected, virtually disappearing from the spectrum, as shown in Figure 7. Of the two others, the one further upfield is somewhat reduced in its intensity. As Figure 7 demonstrates, there are three new signals apparent in the nitrotyrosyllysozyme that were not present before nitration. The resonance positions of the three are totally consistent with those published for 3-nitrotyrosine itself.[16] This strongly suggests that the nitrotyrosine being monitored is fully exposed to the environment.

Discussion

The most important result of this study is the observation of two distinguishable NO_2 stretching modes at 1340 and 1328 cm^{-1} in the resonance Raman spectrum of nitrotyrosyllysozyme. This is a clear advantage of the technique compared with absorption spectroscopy, where only a featureless contour (λ_{max} depending on pH) is noted.

The observation of a solvent-induced shift in the NO_2 stretching frequency of nitrotyrosine permits a decision as to which of the observed features in nitrotyrosyllysozyme arises from a residue in a "normal" environment. Nitrotyrosine in water has a NO_2

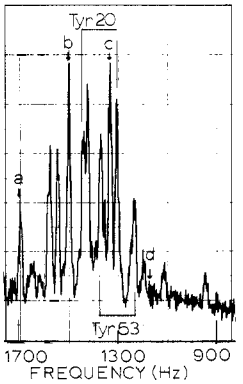

Figure 7. 500-MHz ^1H NMR spectrum of 5.1 mM nitrolysozyme at pH_{app} 4.56 in ^2H_2O recorded at 54 °C; 1000 transients were employed and Fourier transformed after application of the convolution difference technique. Position d denotes the missing Tyr-23 resonance; a, b, and c are new peaks at 8.00, 7.60, and 7.26 ppm, respectively, corresponding to the nitrotyrosine-23 resonances. The frequency is measured downfield from 4.6 ppm.

Raman band at 1342 cm^{-1} and a derived pK_a of 6.74 ± 0.05. These values are within experimental error of those for the high-frequency feature in the resonance Raman spectrum of nitrotyrosyllysozyme (frequency 1340 cm^{-1}; pK_a = 6.76 ± 0.05), suggesting that the tyrosine residue giving rise to this feature is in a normal (unperturbed aqueous) environment. We suggest below that of the three tyrosine residues in the protein (20, 23, and 53), it is tyrosine-23 that produces the 1340-cm^{-1} Raman band upon nitration.

By contrast, the band at 1328 cm^{-1} clearly reflects an unusual environment. the pK_a derived from the titration curve is 6.52 ± 0.05. While this is only 0.2 or 0.3 unit lower than the pK_a for nitrotyrosine in water, the nitration shift of the pK_a for this second tyrosine is significantly larger than that for tyrosine-23. The pK_a for the second tyrosine is at least 11 prior to nitration and 6.5 upon nitration (nitration shift of 4.5 pK_a units), while tyrosine-23 has a nitration shift of 3.2 units.

The model studies (see Results) measuring the solvent dependence of the position of the NO_2 stretching frequency for 3-nitrotyrosine imply that the 12-cm^{-1} shift observed for the second nitrotyrosine in the protein is the result of at least two factors: lower dielectric constant (compare cyclohexane to water) and strong hydrogen bonding (compare $CHCl_3$ to cyclohexane). A further possible factor is the presence of LiOH in the $CHCl_3$ and hexane solutions of the model compound. The effect of ion pairing (which takes place in organic solutions of LiOH) might induce a frequency shift in the NO_2 group of nitrotyrosine of unknown direction or magnitude. Further studies in this area are indicated. Nevertheless, the large shift observed in $CHCl_3$ does strongly implicate a hydrogen-bonding shift.

It is of interest in the current study to determine which residues have been modified. There is general agreement in the literature that of the three tyrosines in lysozyme (20, 23, and 53), 23 is the most reactive. High-field ^1H NMR studies by the Oxford group[11-14] have resulted in an assignment of particular spectral features to tyrosine-23 as indicated in Figures 5 and 6. Upon nitration, these features vanish and are replaced by three new peaks which are at resonance frequencies appropriate for a nitrotyrosine moiety exposed completely to water.[16] The NMR data at low pH are thus consistent with the Raman observation of a spectral feature at 1340 (derived pK_a = 6.76) in that both techniques indicate a residue exposed to the aqueous milieu. The NMR data further suggest that (nitro)tyrosine-23 is responsible for these spectral features.

In contrast to the clear agreement as to the location of the most reactive tyrosine in the protein, there appears to be disagreement as to the location of the second most reactive residue. Atassi and Habeeb[4] employed iodination and nitration to modify tyrosine residues in lysozyme. The nitration experiments were interpretated to mean that tyrosine-20 was modified along with tyrosine-23.

(14) Dobson, C. M.; Ferguson, S. J.; Poulsen, F. M.; Williams, R. J. P. *Eur. J. Biochem.* **1978**, *92*, 99–103.
(15) Campbell, I. D.; Dobson, C. M.; Williams, R. J. P.; Wright, P. E. *FEBS Lett.* **1975**, *57*, 96–99.
(16) Snyder, G. H.; Rowan, R., III; Karplus, S.; Sykes, B. D. *Biochemistry* **1975**, *14*, 3765–3777.

Those results imply that tyrosine-20 is the second most reactive. Our results, in conjunction with the NMR results of the Oxford group,[11-14] suggest that the second most reactive tyrosine (as judged by the decrease in intensity of the resonances upon nitration) is 53. Our NMR data for nitrotyrosyllysozyme suggest that about 30-40% of the tyrosine-53 molecules have been nitrated and that tyrosine-20 has not been modified. The above discussion, of course, depends on the validity of the NMR assignments that were based on Gd(III)-induced broadening. Since X-ray studies have been published for the lysozyme-Gd complex[17] and since tyrosine-53 (of the three available) is the only one near the position of the two Gd(III) ions being bound, we accept the NMR assignments. Three possibilities exist: (i) the chemical modification study assigned 20 instead of 53;[4] (ii) ^{1}H NMR assignment of 20 and 53 is indeed reversed; and (iii) less likely, the nitration in this study modified tyrosine-53; that by Atassi and Habeeb,[4] tyrosine-20. We feel that the third possibility is extremely unlikely; unfortunately our results cannot distinguish between the first and second choices. It is abundantly clear from both the resonance Raman and ^{1}H NMR data that the most reactive tyrosine is in an aqueous environment. The resonance Raman data further indicate that the second tyrosine to be modified is in a very hydrophobic and strongly hydrogen-bonded environment. The utility of resonance Raman spectroscopy for discerning the nature of the environment around tyrosine residues is demonstrated.

(17) (a) Kurachi, K.; Sieker, L. C.; Jensen, L. H. *J. Biol. Chem.* **1975**, *250*, 7663-7667. (b) Perkins, S. J., Johnson, L. W., Machin, P. A.; Phillips, D. C. *Biochem. J.* **1979**, *181*, 21-36.

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Macromonocyclic Polyamines as Biological Polyanion Complexons. 2.[1] Ion-Pair Association with Phosphate and Nucleotides

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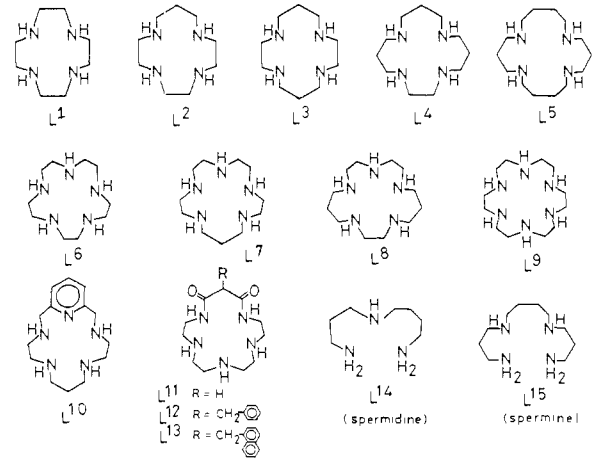
Abstract: The interaction of 15- to 18-membered macromonocyclic tetra-, penta-, and hexaamines with biologically important polyanions, such as phosphate, AMP, ADP, and ATP, in neutral pH solutions has been studied using polarographic methods and ^{1}H NMR shift measurements. The results established 1:1 ion pairings between the polycationic forms of the macrocycles and the polyanionic forms of the phosphates. The inorganic phosphate anion is bound to the macrocycles as strongly as the previously studied polycarboxylate anions. The greater stability constants β_L for the 1:1 nucleotide complexes imply the presence of other binding sites, such as adenine moieties, in the nucleotide molecules. These β_L values are greater than those reported for the nucleotide complexes of physiological linear polyamines (e.g., spermidine and spermine) or of divalent metal cations (e.g., Mg^{2+}). The macrocyclic polyamines, moreover, interact with Mg^{2+}- and Ca^{2+}-nucleotide complexes to yield the ternary complexes. Significant biological implications are discussed.

Introduction

Recognition and binding of ionic substrates to organic host molecules (e.g., enzymes, antibodies, or membrane transporters) are of vital importance in biological reactions. Model studies so far have centered mainly on the recognition of cations using synthetic host compounds, such as macrocyclic polyethers[3-8] and polyamines.[9,10]

Chart I. Macromonocyclic Polyamines and Dioxo Polyamines Studied for the Complexation with Phosphate and Nucleotides



Recently, anion complexons have been drawing much attention. The anion receptors possess appropriate anion binding sites, like

(1) Part 1: Kimura, E.; Sakonaka, A.; Yatsunami, T.; Kodama, M. *J. Am. Chem. Soc.* **1981**, *103*, 3041.
(2) (a) Hiroshima University. (b) Hirosaki University.
(3) Izatt, R. M.; Hansen, L. D.; Eatough, D. J.; Bradshaw, J. S.; Christensen, J. J. In "Metal-Ligand Interactions in Organic Chemistry and Biochemistry", Pullmann, B.; Goldblum, N., Eds.; D. Reidel: Dordrecht, Holland, 1977; pp 337-361.
(4) Ovchinnikov, Yu. A.; Ivanov, V. T.; Shkrob, A. M. In "Membrane-Active Complexones"; Elsevier: New York, 1974; pp 53-72 and 184-198.
(5) Lamb, J. D.; Izatt, R. M.; Christensen, J. J.; Eatough, D. J. In "Coordination Chemistry of Macrocyclic Compounds"; Melson, G. A., Ed.; Plenum Press: New York, 1979.
(6) Izatt, R. M.; Lamb, J. D.; Izatt, N. E.; Rossiter, B. E., Jr.; Christensen, J. J.; Haymore, B. L. *J. Am. Chem. Soc.* **1979**, *101*, 6273-6276, and references therein.
(7) Cram, D. J.; Cram, J. M. *Acc. Chem. Res.* **1978**, *11*, 8-14.
(8) van Bergen, T. J.; Kellogg, R. M. *J. Am. Chem. Soc.* **1977**, *99*, 3882-3884.

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