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Investigation of Solvent Accessibility of the Fluorotyrosyl Residues of M13 Coat Protein in Deoxycholate Micelles and Phospholipid Vesicles†

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ABSTRACT: We have utilized a nonperturbing nuclear magnetic resonance technique, specifically measuring sensitivity of the chemical shift of fluorotyrosyl residues to change in solvent from H_2O to D_2O, to demonstrate that the tyrosyl residues of fluorotyrosyl M13 coat protein in phospholipid vesicles are not accessible to solvent i.e., are buried in the hydrophobic portion of the bilayer. The two fluorotyrosyl residues of the protein *did* show partial exposure to solvent (42% and 65% with respect to aqueous *m*-fluorotyrosine) when the protein was incorporated into deoxycholate micelles, pointing to differences in conformation of micellar protein with respect to vesicle-associated protein. M13 coat protein in phospholipid vesicles was not sensitive to lactoperoxidase-catalyzed iodination, supporting the NMR results. Coat protein in deoxycholate micelles showed release of fluorotyrosyl residues upon Pronase digestion, but only after an observed change in environment. The observed changes suggest that proteolytic digestion studies of membrane proteins should be interpreted with the possibility of artifacts related to conformational changes in mind. M13 coat protein in phospholipid vesicles did *not* demonstrate release of fluorotyrosine by Pronase, again pointing to differences between protein in micelles and in vesicles and corroborating the NMR result.

The nonlytic, filamentous coliphage M13 offers an excellent model system for the study of membrane–protein interactions. The process of phage assembly takes place entirely within the inner membrane of the *Escherichia coli* host, and the major coat protein (gene 8) is stored during infection as a cytoplasmic membrane spanning protein (Smilowitz et al., 1972; Smilowitz, 1974; Wickner, 1976). The coat protein is a 50 amino acid polypeptide of known sequence (Figure 1) and its physical properties have been extensively characterized (Knippers & Hoffmann-Berling, 1966). In addition, it is easily prepared in large quantities from intact phage, which are recovered from the growth medium.

In a previous publication (Hagen et al., 1978), we described the in vivo preparation of a fluorotyrosyl derivative of M13 coat protein and also a method for incorporation of high levels of this protein into small, uniformly sized synthetic phospholipid vesicles. Using high-resolution fluorine-19 nuclear magnetic resonance spectroscopy, we showed that the fluorotyrosyl probe was sensitive to the lipid phase state and proposed a physical model for the motional properties of the fluorotyrosine side chains in the phospholipid bilayer. The model which we proposed was characterized by correlation times for rotation about the $\alpha\beta$ and $\beta\gamma$ bonds of the fluorotyrosyl residues of 50×10^{-9} and 2×10^{-9} s, respectively, indicating increasing mobility away from the polypeptide backbone. Further, to account for all the features of the observed relaxation of the ^{19}F probe, we had to invoke rapid intermolecular fluorine–proton dipolar interactions. Based on the primary sequence (Figure 1) and on studies by other workers involving proteolytic digestion of M13 coat protein in deoxycholate micelles (Woolford & Webster, 1975), we hypothesized that the fluorotyrosyl residues were interacting with lipid methylene protons rather than solvent and estimated that these lipid protons had a minimum lateral diffusion coefficient of 3×10^{-9} cm^2 s^{-1}.

In this manuscript, we demonstrate that fluorotyrosyl residues of M13 coat protein *are* buried in the hydrophobic portion of the bilayer and present the application of a nonperturbing NMR[1] approach to study the exposure of individual

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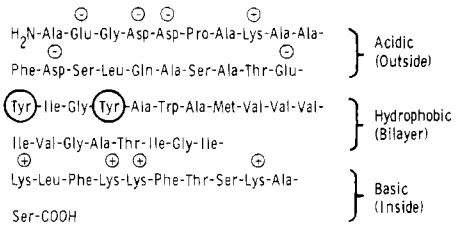

FIGURE 1: The amino acid sequence of M13 coat protein. The sequence is characterized by a hydrophobic central region, an acidic amino terminus, and a basic carboxy terminus (Asbeck et al., 1969; Nakashima & Konigsberg, 1974). The fluorotyrosyl-labeled residues are encircled and reside in the hydrophobic portion of the sequence.

membrane protein residues. In addition, we compare the behavior of coat protein in deoxycholate micelles and in phospholipid vesicles with respect to proteolytic digestion and suggest cautions regarding interpretation of such experiments.

A preliminary report of portions of the solvent isotope shift result has been presented at a symposium in honor of Mildred Cohn (Hagen et al., 1979).

Experimental Procedure

Materials. Sodium deoxycholate was obtained from Sigma Chemical Co. (St. Louis, MO), the lactoperoxidase (50 U/mg) was from Miles Laboratories (Elkhart, IN), the $Na^{125}I$ (50 mCi/mL, carrier free) was from the Edmonton Radiopharmaceutical Center, Edmonton, Alberta, and the Pronase (grade B, 45 000 PKU units/mg) was from Calbiochem (La Jolla, CA). All other materials were as described previously (Hagen et al., 1978).

Growth of Fluorotyrosyl M13 Phage. Fluorotyrosyl M13 phage was prepared exactly as described previously (Hagen et al., 1978).

Isolation of Fluorotyrosyl Coat Protein and Preparation of Deoxycholate Micelles Containing Fluorotyrosyl Coat Protein. Two alternate procedures were employed to isolate the coat protein from whole phage. The first was a phenol extraction carried out exactly as described previously (Hagen et al., 1978). The second was a modification of a deoxycholate–chloroform extraction procedure, recently described by Chamberlain et al. (1978). Briefly, 20 mg of fluorotyrosyl M13 phage was suspended in 1.5 mL of 10 mM Tris-HCl, pH 8, and mixed with 1.5 mL of 0.1 M $NaHCO_3$, pH 9.0, and 300 μL of chloroform. ^{125}I coat protein, 200 000 cpm (800 000 cpm/μg, lactoperoxidase labeled, from tube 3 in Table I), was added as a tracer, and the mixture was incubated at 37 °C with vigorous shaking for 3 h. The clear sample was then applied to a Sephacryl S-200 SF column (1 × 45 cm) equliibrated and eluted with 0.1 M $NaHCO_3$, 8 mM DOC, pH 9.0. Sixty fractions of 1.2 mL were collected in polystyrene tubes and assayed for γ emission in an LKB 1270 Rackgamma II counter. Those fractions corresponding to the major peak of radioactivity (*fluorotyrosyl and tracer iodotyrosyl M13 coat protein in DOC micelles*) were pooled and concentrated to 1.5 mL in an Amicon ultrafiltration apparatus (PM-10 membrane) for NMR experiments. Different concentrations of D_2O were introduced by resuspending the concentrated sample in a larger volume of 0.1 M $NaHCO_3$, 8 mM DOC, pH 9.0, prepared with various levels of D_2O, and reconcentrating to 1.5 mL.

[1] Abbreviations used: NMR, nuclear magnetic resonance, DOC, sodium deoxycholate; KP_i, buffer prepared with mono- and dibasic potassium phosphate; BSA, bovine serum albumin; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; SIS, solvent isotope shift, TFA, sodium trifluoroacetate.

Preparation of Fluorotyrosyl Coat Protein Containing Vesicles. Vesicles containing fluorotyrosyl M13 coat protein (phenol extracted) were prepared exactly as described in Hagen et al. (1978), except that for NMR experiments the final volume was 1.5 mL and for lactoperoxidase labeling the azide was removed by dialysis.

Lactoperoxidase Labeling. Iodination was carried out by a modification of the Morrison (1974) procedure. A neutralized ^{125}I stock solution was mixed with an equal volume of 10 μM sodium sulfite to reduce any I_2 to I^-. A 1 mg/mL solution of lactoperoxidase was prepared in 0.1 M KP_i, pH 7.5. A stock peroxide solution was prepared by dissolving 3 μL of a 30% hydrogen peroxide solution in 4.5 mL of 0.1 M KP_i, pH 7.5. The reaction mixtures in Table I were set up and allowed to react for 1 h, after which time 50 μL of 0.4% acetic acid and 400 μL of a buffer containing 50 mM sodium acetate, pH 4, 0.5 M NaCl, 1 mg/mL BSA, and 0.2 mg/mL NaN_3 were added. The sample was then dialyzed at 23 °C against the same buffer for 4 days (buffer changed once at 2 days).

Radioactivity incorporated was determined by liquid scintillation counting of β emission in toluene–Trition counting fluid (Weiner & Heppel, 1971) by using a Beckman Model LS-230 liquid scintillation system.

Pronase Digestion. (*a*) *DOC Micelles.* Approximately 10 mg of fluorotyrosyl coat protein in deoxycholate micelles, prepared from 20 mg of phage, as described above, in a final volume of 1.5 mL, was placed in a 10-mm NMR tube. The final concentration of deoxycholate was not assessed due to the ultrafiltration step but was well above the critical micellar concentration for deoxycholate. Ten microliters of a 7.5 mg/mL Pronase solution in 10 mM Tris-HCl, pH 8.0, containing 100 mM NaCl was added and the digestion monitored at 28 °C by ^{19}F NMR spectroscopy.

(*b*) *Vesicles.* Phospholipid vesicles containing approximately 0.2 mg of fluorotyrosyl coat protein, prepared as described above in a final volume of 1.5 mL, were placed in a 10-mm NMR tube, 5 μL of the above Pronase solution was added, and the digestion was monitored at 28 °C by ^{19}F NMR. An additional 25 μL of the Pronase solution was added 15 h after the initial addition.

NMR Methods. All ^{19}F measurements were made at 254 MHz on a Bruker HXS-270 NMR spectrometer, operating in the Fourier transform mode, essentially as described previously (Hagen et al., 1978). All ^{19}F chemical shifts were measured relative to a separate sample of 10 mM TFA in 99% D_2O, pH 7 at 300 K (similarly locked on the ^2H resonance of the solvent). Solvent isotope shifts (SIS) for fluorine measured in this manner do not take into account the shift of the ^2H lock resonance of the sample with changing concentration of D_2O/H_2O. The solvent isotope shift for H_2O, HDO has been measured in acetone (Holmes et al., 1962) and is much smaller (0.030 ppm) than the SIS's reported here. Therefore, this secondary effect will be neglected. In any case, it does not affect the conclusions of this manuscript since the measurements for the fluorotyrosine standard and the protein were made in the same manner. Upfield shifts were taken as negative. The SIS is taken as $\delta_{H_2O} - \delta_{D_2O}$, and, therefore, a positive SIS corresponds to more upfield shifts in D_2O.

Peak positions for the solvent isotope shift experiments were obtained by a parabolic interpolation of the digitalized spectra, using Nicolet Technology Corp. software. Spectra for the same sample of fluorotyrosyl coat protein in vesicles at various levels of D_2O were recorded at a digital resolution of 1.5 Hz/point. These spectra yielded identical (±0.01 ppm) peak

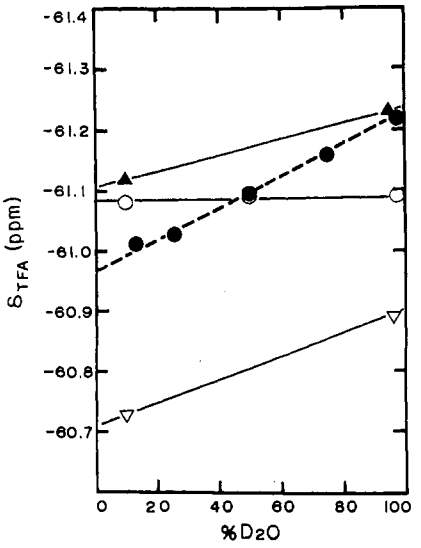

FIGURE 2: ^{19}F NMR chemical shift measured at 254 MHz vs. 10 mM trifluoroacetic acid in D_2O, pH 7, for the following. (O—O) Fluorotyrosyl M13 coat protein in synthetic phospholipid vesicles, in 50 mM KCl, 10 mM Tris-HCl, 1 mM EDTA, 0.02% NaN_3, at pH 8.1, 300 K; (●---●) 2 mM *m*-fluorotyrosine in 50 mM KCl, 25 mM Tris-HCl, 1 mM EDTA, at pH 8, 300 K, and fluorotyrosyl M13 coat protein in deoxycholate micelles in 0.1 M $NaHCO_3$, pH 9, 300 K; (▽—▽) is peak 1 in Figure 3A and (▲—▲) is peak 2 (upfield peak).

positions. As a measure of the precision of the interpolation method itself, six spectra, which had been recorded at lower digital resolution (6 Hz/point) during a T_1 experiment, were analyzed. The average deviation in peak position was ±0.03 ppm, corresponding to 12% of the maximum expected solvent isotope shift (see Results). We thus make the conservative assertion that our solvent isotope shift determinations (at higher digital resolution) are capable of detecting solvents shifts which are 20% of the maximum observed shift.

Results

Solvent Isotope Shift. The ^{19}F resonance position of *m*-fluorotyrosine in aqueous solution has been demonstrated to be dependent upon whether the solution is prepared with H_2O or D_2O, typically shifting about 0.25 ppm upfield as one proceeds from 0 to 100% D_2O (Hull & Sykes, 1976; Lauterbur et al., 1978). This property, which derives from hydrogen-bonding interactions between the fluorine atom and solvent, has been utilized to determine the degree of accessibility to solvent of tyrosyl residues in a fluorotyrosyl derivative of alkaline phosphatase (Hull & Sykes, 1976). It was found in that study that fluorotyrosyl residues which appeared to be least exposed on the basis of two other criteria, the chemical shift and the relaxation rate due to nonring protons, demonstrated a lower solvent isotope shift.

We have applied this essentially nonperturbing approach in a new context to determine the degree of exposure to solvent of fluorotyrosyl residues of M13 coat protein in deoxycholate micelles and in phospholipid vesicles. The results of this investigation are presented in Figure 2. Aqueous *m*-fluorotyrosine (pH 8.0) showed a solvent isotope shift ($\delta_{H_2O} - \delta_{D_2O}$) of 0.26 ppm and is taken to be fully exposed to solvent. Fluorotyrosyl M13 coat protein in deoxycholate micelles produced an ^{19}F spectrum characterized by two major peaks (Figure 3A, peaks 1 and 2), which we believe correspond to the two tyrosyl residues in the coat protein sequence. These peaks demonstrated SIS values of 0.17 ppm (peak 1) and 0.11 ppm (peak 2), indicating both residues are partially exposed to solvent. We estimate on this basis that one residue is 65% and the other 42% exposed with respect to aqueous *m*-fluorotyrosine.

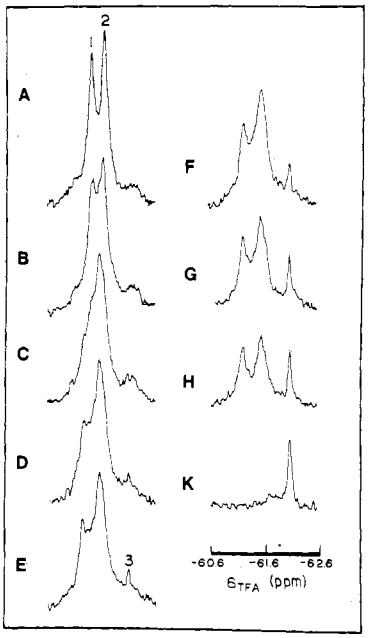

FIGURE 3: Pronase digestion of fluorotyrosyl M13 coat protein in deoxycholate micelles. This figure presents representative spectra from various times during the course of the digestion; each spectrum is a time block of 16.25 min. Spectrum A is a control spectrum, recorded before addition of Pronase; spectrum B was recorded from 0 to 16 min, C from 32 to 49 min, D from 65 to 81 min, E from 114 to 130 min, F from 146 to 162 min, G from 211 to 228 min, H from 292 to 309 min, and K after 3 days of digestion. The labeled peaks and spectral changes are discussed in the Results section. The scaling bar indicates chemical shifts vs. 10 mM TFA in D_2O, pH 7.

When the fluorotyrosine-labeled coat protein was incorporated into phospholipid vesicles with the cholate–urea dialysis procedure, the observed solvent isotope shift was zero. We estimate that we would detect a solvent isotope shift of as little as 20% of that of aqueous fluorotyrosine, and, as the protein is too small for folding to likely be a factor, we infer that the fluorotyrosyl residues are experiencing little or no interaction with solvent, that is, are buried in the hydrophobic region of the phospholipid bilayer.

Lactoperoxidase Labeling. Because the SIS experiment represents a relatively new approach, we thought it necessary to corroborate our result with two more commonly employed techniques. The first was lactoperoxidase-catalyzed iodination of tyrosyl residues, and the other was Pronase digestion of M13 coat protein in deoxycholate micelles and vesicles.

Lactoperoxidase is capable of catalyzing direct iodination of tyrosyl residues, but in the absence of saturating levels of iodinatable substrate it is also capable of catalyzing the conversion of I^- to I_2. Molecular iodine can then freely enter the bilayer and add onto lipid double bonds or protein residues (Morrison, 1974). We had a unique problem in this regard since the tyrosyl residues could be buried in the phospholipid bilayer and not exposed to the enzyme but still be iodinated by I_2 diffusing into the bilayer. To qualitatively assess this effect, we included a reaction mixture containing aqueous tyrosine in addition to the vesicle sample to provide the enzyme with iodinatable substrate.

The results of the labeling experiment are presented in Table I. The reaction mixture containing aqueous tyrosine (tube 1, ratio of protein tyrosyl to aqueous tyrosine 175 to 1) was labeled to a specific activity of only 0.3% that of a suspension of coat protein in buffer, indicating no exposure of protein tyrosyl residues in the vesicles to labeling. However, lacto-

Table I: Lactoperoxide-Catalyzed Iodination of M13 Coat Protein in Phospholipid Vesicles[a]

tube no.	components	concn (mM)	cpm	sp act. (%)
1	24 μL of ^{125}I (0.4 mCi)	2×10^{-3}	1.4×10^{6}	0.3
	50 μL of vesicle sample	protein: 1.2		
	(0.5 mg of protein)	tyrosyl: 2.5		
	1 μL of lactoperoxidase	2.0×10^{-4}		
	1 μL of 0.02% L-Tyr, pH 1.0	1.4×10^{-2}		
	1 μL of peroxide	8.0×10^{-2}		
2	(as for tube 1, but no Tyr)		9.7×10^{6}	2.4
3	24 μL of ^{125}I	2.0×10^{-3}	40.2×10^{6}	100.0
	50 μL of M13 coat protein	1.3×10^{-1}		
	(1 mg/mL in 0.1 M KP_i, pH 7.5)			
	1 μL of lactoperoxidase	2.0×10^{-4}		
	1 μL of peroxide	8.0×10^{-2}		

[a] Solutions are described in the Experimental Procedure section and were added in the order in which they are listed.

peroxidase may have a much greater affinity for aqueous tyrosine than for protein tyrosyl residues, and the result for the vesicle sampling lacking aqueous tyrosine (tube 2, Table I) offers more convincing evidence. In this case, which represents nonsaturation of lactoperoxidase with iodinatable substrate, the specific activity was only 2.4% that of the aqueous coat protein suspension. Thus, lactoperoxidase labeling supports the SIS result in that the tyrosyl residues of M13 coat protein in phospholipid vesicles are not accessible to enzyme-catalyzed iodination.

Pronase Digestion. Deoxycholate-solubilized fluorotyrosyl M13 coat protein was prepared as described above and subjected to Pronase digestion, with the progress of the digestion followed by ^{19}F NMR spectroscopy. The spectra for the digestion are presented in Figure 3.

Spectrum A is a control spectrum recorded before addition of Pronase and is characterized by two major peaks (1 and 2). Spectrum K was recorded 3 days after addition of Pronase and is dominated by a single peak (no. 3) in the position expected for aqueous fluorotyrosine or a small fluorotyrosine-containing peptide. The area of peak 3 appearing in any of spectra B to K is much less than the amount by which the combined area of peaks 1 and 2 has been reduced in the same spectrum relative to their area in spectrum A. This occurs because the spin–lattice relaxation time is much longer for the released tyrosyl peak no. 3 than for the DOC micelle tyrosyl peaks no. 1 and 2. While the relative areas of peak 1 or 2 vs. peak 3 are not accurate for a given spectrum, the relative areas of any given peak between different spectra are accurate (Sykes & Hull, 1978). Clearly, proteolysis of fluorotyrosyl M13 coat protein in deoxycholate micelles yields complete release of tyrosyl residues from the hydrophobic region of the protein.

The course of the Pronase digestion was very interesting and was characterized by extensive changes in chemical environment of the fluorotyrosyl residues prior to their cleavage. Peak 1, which demonstrates the greatest exposure on the basis of SIS, initially migrated upfield upon addition of Pronase, suggesting a change of environment as residues in the hydrophilic termini were removed. Peak 2, which corresponds to the more buried residue, migrated slightly downfield, displaying the same sensitivity to removal of more distal residues. At a later time, the "combined" peak (spectrum C) again splits into two resonances (in *new* positions relative to peaks 1 and 2) whose intensities thereafter decreased monotonically with time. While this was occurring, the "free" peak (peak 3) was gradually rising in intensity, first appearing in the spectrum after about 60 min of digestion. Thus the Pronase digestion was characterized by extensive changes in environment of the fluorotyrosyl residues prior to the onset of, and during the course of, their cleavage.

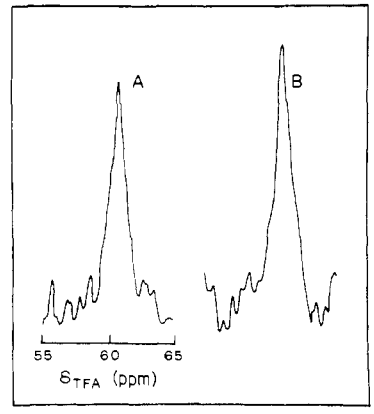

FIGURE 4: Pronase digestion of fluorotyrosyl M13 coat protein in phospholipid vesicles. Spectrum A was recorded from 0 to 2.2 h and spectrum B from 38.2 to 40.5 h after initial addition of Pronase. Both are identical with ^{19}F spectra previously obtained for this system (Hagen et al., 1978). The scaling bar indicates chemical shift vs. 10 mM TFA in D_2O, pH 7.

These results are to be contrasted with those for fluorotyrosyl M13 coat protein in phospholipid vesicles. The spectra in Figure 4 represent a control spectrum and a spectrum recorded after about 40 h of Pronase digestion. There was no effect whatsoever on the spectrum; the fluorotyrosyl residues were *not* susceptible to cleavage by Pronase. Clearly, the behavior of this protein in deoxycholate micelles is very different from that observed in phospholipid vesicles.

Discussion

In a previous analysis of the spectral properties of fluorotyrosyl M13 coat protein in phospholipid vesicles (Hagen et al., 1978), we invoked rapid intermolecular fluorine–proton dipolar interactions to account for the dipolar contribution to the observed spin–lattice relaxation time T_1. These intermolecular fluorine–proton interactions were attributed to the methylene groups of lipid fatty acyl chains. This assignment was made on the basis of the primary sequence of the protein (which suggests that the tyrosyl residues would be in the portion of the protein spanning the bilayer) and the previously demonstrated inaccessibility of tyrosyl residues of M13 coat protein in deoxycholate micelles to chymotryptic or tryptic cleavage (Woolford & Webster, 1975). We had further support for this hypothesis in that we observed a negative nuclear Overhauser effect when irradiating lipid methylene protons, although we noted that this effect *could* possibly be indirectly mediated by the protein were the fluorotyrosyl residues exposed to solvent. We felt it necessary to examine this question in greater detail and utilized three approaches

to do so: an NMR solvent isotope shift experiment, lactoperoxidase-catalyzed iodination, and Pronase digestion.

The fluorine nucleus in a fluorotyrosine ring is susceptible to a solvent isotope shift; that is, its resonance position is sensitive to whether H_2O or D_2O is the bathing solvent (Lauterbur et al., 1978; Hull & Sykes, 1976). This effect derives from hydrogen-bonding interactions between the fluorine atom and the solvent and typically has a magnitude of about 0.25 ppm for aqueous fluorotyrosine, which is taken as fully exposed. Our observed solvent isotope shift for *m*-fluorotyrosine was 0.26 ppm, and this was utilized as a "fully exposed" standard. Fluorotyrosyl M13 coat protein in deoxycholate micelles is characterized by two major ^{19}F NMR peaks (Figure 3A) which demonstrate SIS values of 0.17 ppm (peak 1) and 0.11 ppm (peak 2), indicating relative exposure of 65%, and 42%, respectively. This "partial exposure" does not necessarily mean that the fluorotyrosyl residues protrude from the deoxycholate micelle. Deoxycholate is a rigid steroid ring, and interposition of a protein molecule with bulky side chains may allow for considerable entry of solvent water into the micellar domain.

Fluorotyrosyl M13 coat protein in phospholipid vesicles, however, did *not* demonstrate a solvent isotope shift. We conclude that the tyrosyl residues of coat protein in vesicles are not experiencing significant exposure to aqueous solvent. With respect to the suggestion by Chamberlain et al. (1978) that tyrosyl residues would occasionally enter the aqueous region by way of protein motion, we estimate that we could detect as little as 20% exposure to solvent.

We think that this nonperturbing approach will find widespread application in investigating "buriedness" of membrane protein residues. Fluorotyrosyl derivatives of proteins are relative easily prepared in vivo, and the NMR experiment itself is straightforward. One possible problem for larger proteins is folding, which could yield results suggesting that a residue is buried in the membrane when it is actually in a hydrophobic pocket of the protein.

Lactoperoxidase labeling has been utilized to determine orientation and asymmetry of membrane proteins in red blood cells and other systems (see Morrison, 1974). This enzyme possesses the unfortunate property of catalyzing conversion of I^- to I_2 in the absence of saturating levels of directly iodinatable substrate, such as tyrosine. As the possibility existed that tyrosyl residues were not accessible to the enzyme, we included for our study a reaction mixture which contained aqueous tyrosine in addition to the coat protein-containing vesicle sample (see Results). We found that M13 coat protein in phospholipid vesicles was *not* susceptible to lactoperoxidase-catalyzed iodination, as expected from the SIS result.

Pronase digestion of fluorotyrosyl M13 coat protein in deoxycholate micelles yielded very interesting results. All of the fluorotyrosine was released from the hydrophobic core, as might be expected from its observed "exposure" to solvent, but the pattern of digestion was far more informative (Figure 3). It was found that the chemical environment of the residues changed dramatically prior to and during the course of the digestion. The peak which demonstrated the greatest exposure to solvent, for example, moved upfield prior to release of "free" fluorotyrosine, indicating an apparent adjustment of environment in response to removal of residues in the hydrophilic termini. Only after 60 min of digestion did detectable amounts of free fluorotyrosine (peak 3 in Figure 3) appear. These extensive conformational changes would certainly be missed were one to follow amino acid release, for example, and point to a very serious problem with proteolytic digestion of membrane proteins. It must now be considered necessary to establish that residues released in a proteolytic digestion have *not* been released because of earlier conformational changes, or at least to consider this possibility in interpretation of a digestion experiment. Again, ^{19}F NMR suggests itself as a valuable tool in following the course of such digestions. Fluoro derivatives of other amino acids are commercially available and, in conjunction with fluorotyrosine, offer the potential of yielding much more extensive and reliable information from digestion experiments.

Coat protein in phospholipid vesicles, by way of contrast, did *not* demonstrate release of fluorotyrosyl residues upon Pronase digestion, and further a proton NMR spectrum (data not shown) did not indicate appreciable release of free amino acids or small oligopeptides. We are actively investigating this behavior, which points once more to the serious differences between protein in micelles and protein in vesicles.

Summary

We have demonstrated, by means of an NMR solvent isotope shift experiment, that fluorotyrosyl residues of M13 coat protein in phospholipid vesicles are *not* accessible to solvent and have thereby solidified our earlier conclusions (Hagen et al., 1978). This result has been supported by two independent approaches: lactoperoxidase-catalyzed iodination and Pronase digestion.

We are forced to the further conclusion, on the basis of the SIS and Pronase experiments, that proteins in deoxycholate micelles are quite different from protein in vesicles and propose that more serious consideration be given to the single "membrane-bound" conformation suggested by Chamberlain et al. (1978). In addition, our results suggest that proteolytic digestion experiments must be interpreted with appropriate consideration for possible artifacts related to conformational changes occurring during the digestion.

Lastly, the solvent isotope shift experiment we have described suggests itself as one with widespread potential applicability, as it is a nonperturbing means of establishing buriedness of membrane protein residues.

Acknowledgments

The authors thank Elke Lohmeier for preparation of the figures, Dr. John H. Baldo for the development of a 10-mm ^{19}F NMR probe, and Gerard McQuaid for upkeep of the NMR spectrometer.

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Properties of *Escherichia coli* 16S Ribosomal Ribonucleic Acid Treated with 4,5′,8-Trimethylpsoralen and Light†

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ABSTRACT: 16S rRNA reacted with the furocoumarin 4,5′,8-trimethylpsoralen (trioxsalen) and 360-nm light showed a number of chemical and physical differences from untreated RNA. After extensive irradiation, five molecules of trioxsalen were bound per molecule of RNA. The trioxsalen-treated RNA had an altered ultraviolet absorption spectrum and a distinctive fluorescence emission spectrum. The modified RNA was significantly more resistant to T_1 ribonuclease digestion than was control RNA. Treated RNA, when mixed with purified ribosomal proteins, was not functional in the in vitro reconstitution of 30S subunits and yielded more slowly sedimenting particles which were inactive in protein synthesis assays. By contrast, 16S rRNA within the 30S subunit structure did not exhibit these changes when reacted with the same dose of trioxsalen and light, suggesting that the ribosomal proteins were effective in protecting the RNA from interaction with the drug.

Specific secondary and tertiary structures of rRNA are important in the formation, structure, and function of ribosomes (Zimmermann, 1974; Kurland, 1977). In particular, the 16S rRNA from *Escherichia coli* has been described as a flexible molecule whose conformation in solution can be altered by the ionic environment (Cox & Littauer, 1962), temperature (Schulte et al., 1974), or ribosomal proteins (Sypherd, 1971; Seals & Champney, 1976; Hochkeppel & Craven, 1976, 1977).

The complete in vitro reassembly of 30S subunits (reconstitution) takes place after a substantial conformational change of the 16S RNA has occurred (Held & Nomura, 1973). At low temperatures a protein-deficient reconstitution intermediate (RI) particle is formed. Heat activation of this 21S RI particle produces a more compacted 26S particle (RI*), with the same protein composition. This RI to RI* transition has recently been interpreted as an open to closed transformation of the tertiary structure of the 16S rRNA (Hochkeppel & Craven, 1977).

Little work has been done regarding other possible changes in the secondary or tertiary structure of the 16S RNA during the in vitro assembly process (Bollen et al., 1970). The identification of specific 16S RNA structural states during assembly is made difficult by the observation that these states are generally maintained only in the presence of certain ribosomal proteins. Deproteinization of a specific ribonucleoprotein intermediate can lead to a randomization of the particular RNA structural state.

Psoralen derivatives have been used to stabilize nucleic acid structures. These furocoumarins intercalate into nucleic acids, forming single-strand monoadducts and interstrand cross-links in single- or double-stranded DNA and RNA molecules (Rodighiero et al., 1970; Cole, 1971; Isaacs et al., 1977). The equilibrium binding constant for psoralen–nucleic acid complex formation is affected by such factors as the extent of secondary structure present in the nucleic acid, the type of psoralen derivative used, and the ionic strength and temperature of the solution (Dall'Acqua & Rodighiero, 1966; Dall'Acqua et al., 1969). The latter variables seem mainly to affect the structural state of the nucleic acid but not the photochemical reaction itself. Increasing the ionic strength of a nucleic acid solution (Shen & Hearst, 1976) or decreasing the temperature (Dall'Acqua et al., 1969) will result in a more extensive secondary (and tertiary) structure in the molecule. Consequently, the formation of molecular complexes between psoralens and nucleic acids would be expected to increase under these conditions (Rodighiero et al., 1970). For a particular nucleic acid, under fixed conditions of temperature, ionic strength, and light dose, the extent of drug photobinding should reflect the structural status of the nucleic acid in its specific environment. Thus, psoralens can be used not only to stabilize nucleic acid structure but also as a probe for detecting differences in the nucleic acid configuration in specific environments.

Recently, the stabilization of both double- and single-stranded DNAs (Cech & Pardue, 1976; Shen & Hearst, 1976) and RNAs (Hearst & Thiry, 1977; Issacs et al., 1977) from several sources has been reported. These observations, in combination with the demonstrated binding of 8-methoxypsoralen to *E. coli* transfer RNA (Ou & Song, 1978), suggested the possibility of using trioxsalen[1] to stabilize 16S rRNA secondary structure. In this communication, we describe the use of trioxsalen treatment as a technique for fixing the structure of 16S rRNA, in the presence and absence of 30S ribosomal proteins. The relative nonreactivity of the RNA

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[1] Abbreviations used: trioxsalen, 4,5′,8-trimethylpsoralen; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; A_{260}, absorbance at 260 nm of a 1-mL solution in a 1-cm light path.

0006-2960/79/0418-2012$01.00/0