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Novel Tyrosine Markers in Raman Spectra of Wild-Type and Mutant (Y21M and Y24M) *Ff* Virions Indicate Unusual Environments for Coat Protein Phenoxyls†

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ABSTRACT: The tyrosine side chain generates a pair of distinctive Raman bands—a Fermi doublet near 850 and 830 cm $^{-1}$—with relative intensities diagnostic of hydrogen bonding states of the phenolic acceptor and donor atoms [Siamwiza et al. (1975) *Biochemistry 14*, 4870–4876]. This structural correlation has been tested extensively and is used widely as an indicator of tyrosine interactions in globular proteins and their assemblies. However, in *Ff* filamentous viruses (*fd*, *f1*, *M13*) the apparent Fermi doublet intensity ratio ($I_{853}/I_{826} \approx 4.0$) is much greater than the maximum predicted or observed in other proteins. To understand this anomaly, we have reevaluated the basis for the Fermi doublet assignment in *Ff*. We report Raman spectra of site-specific mutants of *Ff* in which either one (Y21M and Y24M) or both (Y21F/Y24S) tyrosines of the coat protein subunit (pVIII) have been mutated. These Raman data, together with those obtained from *Ff* virions carrying residue-specific tyrosyl (Y-*d4*) and phenylalanyl (F-*d5*) deuterations in pVIII, demonstrate conclusively that the 853 and 826 cm^{-1} bands of *Ff* do not constitute a typical tyrosine Fermi doublet: *The observed 826 cm^{-1} Raman band of Ff is due not to tyrosine but to phenylalanine residues of pVIII. The 853 cm^{-1} Raman band thus constitutes the first known example of a "tyrosine singlet" in the Raman spectrum of a protein.* The implications of this finding for *Ff* virion structure and its relevance to tyrosine markers in other proteins are discussed.

The filamentous coliphage *Ff* (*fd*, *f1*, *M13*) packages a single-stranded DNA genome of ≈6400 nucleotides within a cylindrical sheath comprising ≈2700 copies of a 50-residue α-helical coat protomer (pVIII) and a few copies of minor proteins at the filament ends (Denhardt et al., 1978). The pVIII subunits constitute about 87% of the total mass or 98% of the protein mass of the mature virion. The amino acid sequence of pVIII (1AEGDDPAKAAFDSLQASATEYI-GYAWAMVVVIVGATIGIKLFKKFTSKAS50) is identical in *fd* and *f1*; in *M13*, D12 is replaced by N12. The importance of *Ff* as a cloning vector, the prospects for use of its protein coat in antibody display and the intriguing pathway of virion assembly at the host cell membrane have combined to make *Ff*, and especially pVIII, a target of intensive structural analysis by spectroscopic and fiber diffraction methods. [For reviews and recent applications, see Model and Russel (1988), Russel (1991, 1993), Cesareni (1992), Marks et al. (1992), Aubrey and Thomas (1991), Miura and Thomas (1994), Glucksman et al. (1992), Opella and McDonnell (1994), and McDonnell et al. (1993), Marvin et al. (1994)].

The exploitation of molecular spectroscopy for structural study of filamentous viruses requires definitive assignment of spectral bands to specific residues of viral coat protein and DNA constituents. Reliable assignments have been accomplished for many of the Raman bands through systematic comparison of Raman spectra of different filamentous phages of class I (*Ff*, *IKe*, *If1*) and class II (*Pf1*, *Xf*, *Pf3*) symmetries,

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and by analogy with Raman spectra of model compounds (Thomas & Murphy, 1975; Thomas et al., 1983). However, more direct methods are required to resolve problematic assignments (Thomas et al., 1988). Since residue-specific isotope labeling is the method of choice for eliminating ambiguities in Raman vibrational assignments, we recently initiated a detailed study of *Ff* variants incorporating specifically-deuterated side chains into pVIII subunits of the mature virion (Aubrey & Thomas, 1991; Overman & Thomas, 1994). An important objective of these studies has been to elucidate the spectral intensities of the tyrosine Fermi doublet in *Ff* coat protein. The Fermi components, anticipated near 850 and 830 cm^{-1} and expected to be informative of phenolic hydrogen bonding states (Siamwiza et al., 1975), were assigned previously to the pair of bands observed in *Ff* at 853 and 826 cm^{-1} (Thomas et al., 1983).

In the course of these investigations, we have observed that the pair of Raman bands at 853 and 826 cm^{-1} in the spectrum of *Ff* exhibit unexpected behavior upon deuteration of either phenoxyl or phenyl ring sites of the respective tyrosine (Y21, Y24) or phenylalanine (F11, F42, F45) residues in pVIII (Overman & Thomas, 1994). These findings have suggested, remarkably, that the pVIII subunit of the assembled *Ff* virion might not display the anticipated tyrosine Fermi doublet. In the present work we show that elimination of one or both tyrosines by site-directed mutagenesis of pVIII leads convincingly to the conclusion that the coat protomer lacks altogether a typical tyrosine Fermi doublet in the 800–900 cm^{-1} region of its Raman spectrum. The present conclusion impacts upon proposed models for the architecture of subunit assembly in the native *Ff* virion and has broad implications for the classic structural correlation proposed originally by Siamwiza et al. (1975).

MATERIALS AND METHODS

Reagents and Phage. Specifically deuterated amino acids L-phenylalanine-2′,3′,4′,5′,6′-d_5 (F-*d5*) and L-tyrosine-2′,3′,5′,6′-d_4 (Y-*d4*) were obtained from Cambridge Isotope Laboratories (Woburn, MA). Normal amino acids, growth media and standard reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (St. Louis, MO).

Wild type bacteriophage (*fd* and *f1*) and their deuterium isotope derivatives were prepared as follows. The phages were grown in MS^+ media with *Escherichia coli* strain Hfr3300 as host (stocks obtained from Dr. Loren A. Day, Public Health Research Institute, NY). To incorporate a deuterated amino acid into the pVIII coat protein, the host and phage were grown in M9 minimal media containing the appropriate labeled L-amino acid (either F-*d5* or Y-*d4*), the remaining normal L-amino acids, and additional supplements. Complete incorporation (>99%) of the deuterated amino acid side chain(s) was verified as described (Aubrey & Thomas, 1991; Overman & Thomas, 1994).

Mature viral particles, extruded through the bacterial membrane and into the growth media, were collected by poly(ethylene glycol) precipitation followed by low speed centrifugation. The phage particles were purified either on a continuous KBr gradient or by differential centrifugation. Repeated cycles of differential centrifugation in 10 mM Tris (pH 7.8) yielded a pellet from which samples were prepared for spectroscopic examination. In a typical preparative protocol, 1 L of culture yielded 15–25 mg of purified bacteriophage particles at a concentration of 100 mg/mL in a buffer of 10 mM Tris, pH 7.8. Further details of preparation of normal and deuterated isotopomers of *Ff* phage have been described (Aubrey & Thomas, 1991; Overman & Thomas, 1994).

The mutant *f1* phages were constructed by site-directed mutagenesis by hybridizing mutagenic oligonucleotides to a single-stranded DNA template. The oligonucleotides utilized in the mutagenic procedure were R60(CATAACCGATCATTTCGGTCGCT) for the single mutant Y21M, R104(CATCGCCCACGCCATACCGATATATTC) for the single mutant Y24M, and R145(CATCGCCCACGCCATACCGATATATTC) for the double mutant Y21F/Y24S. Mutant clones were identified by hybridization to the mutagenic oligonucleotides at stringent conditions and characterized by sequencing the entire gene VIII.

The mutant phages [*f1*(Y21M), *f1*(Y24M), and *f1*(Y21F/Y24S)] were grown, harvested, purified, and pelleted as indicated above.

Raman Spectroscopy and Data Analysis. A 10 μL aliquot of the final pellet of each phage was transferred to a glass capillary (Kimax no. 34507) employed as the Raman sample cell. The cells were sealed and stored at 4 °C prior to data collection and were thermostated at 12 °C during data collection protocols.

Raman spectra were excited with the 514.5 nm line of an argon laser (Innova-70, Coherent, Santa Clara, CA) and were recorded in the 90° scattering geometry on a scanning spectrophotometer consisting of a sample illuminator and double monochromator assembly (Model V/VI, Spex Industries, Metuchen, NJ) equipped with a cooled photomultiplier detector (Model R928P, Hamamatsu, Middlesex, NJ) under microcomputer control. Further details of the Raman instrumentation have been described (Li et al., 1981).

For the spectral interval 300–1800 cm^{-1}, eight scans were collected repetitively and averaged. For the narrower spectral intervals, 300–515 and 600–900 cm^{-1}, up to 50 scans were averaged. Difference spectra, employed to visualize shifts of Raman bands resulting from deuteration or mutation of side chains, were computed using either SpectraCalc (Galactic Ind., Salem, NH) or Sigma Plot (Jandel Scientific, Corte Madera, CA) software. For computation of difference spectra, Raman intensities of minuend and subtrahend were normalized to eliminate bands which are invariant to the imposed changes of primary structure (Thomas et al., 1983; Aubrey & Thomas, 1991; Overman & Thomas, 1994).

RESULTS

Raman spectra in the region 300–1800 cm^{-1} of the wild type *Ff* virus (*fd*) and single-site mutants, *f1*(Y21M) and *f1*(Y24M), are shown in Figure 1. The intervals 600–900 cm^{-1} from spectra of these three virions are compared on an expanded scale in Figure 2. The same intervals from Raman spectra of normal and isotopically substituted tyrosyl and phenylalanyl derivatives of *Ff* are compared in Figure 3, and Raman spectra in the region 300–515 cm^{-1} of the normal *Ff* virus and its deuteriotyrosine isotopomer are compared in Figure 4. [We use the notation *fd*($2Y_{d4}$) to designate the *fd* variant (isotopomer) assembled from pVIII subunits in which the two tyrosines (Y21 and Y24) per subunit are deuterated at the four phenoxyl ring positions. Similarly, *fd*($3F_{d5}$) designates the isotopomer in which the three phenylalanine residues (F11, F42, and F45) per subunit are deuterated at the five phenyl ring sites.]

In both Figures 2 and 3, difference spectra are included to display Raman band frequency and intensity changes which accompany the respective mutations and deuterium labeling of side chains. The most important spectral changes within

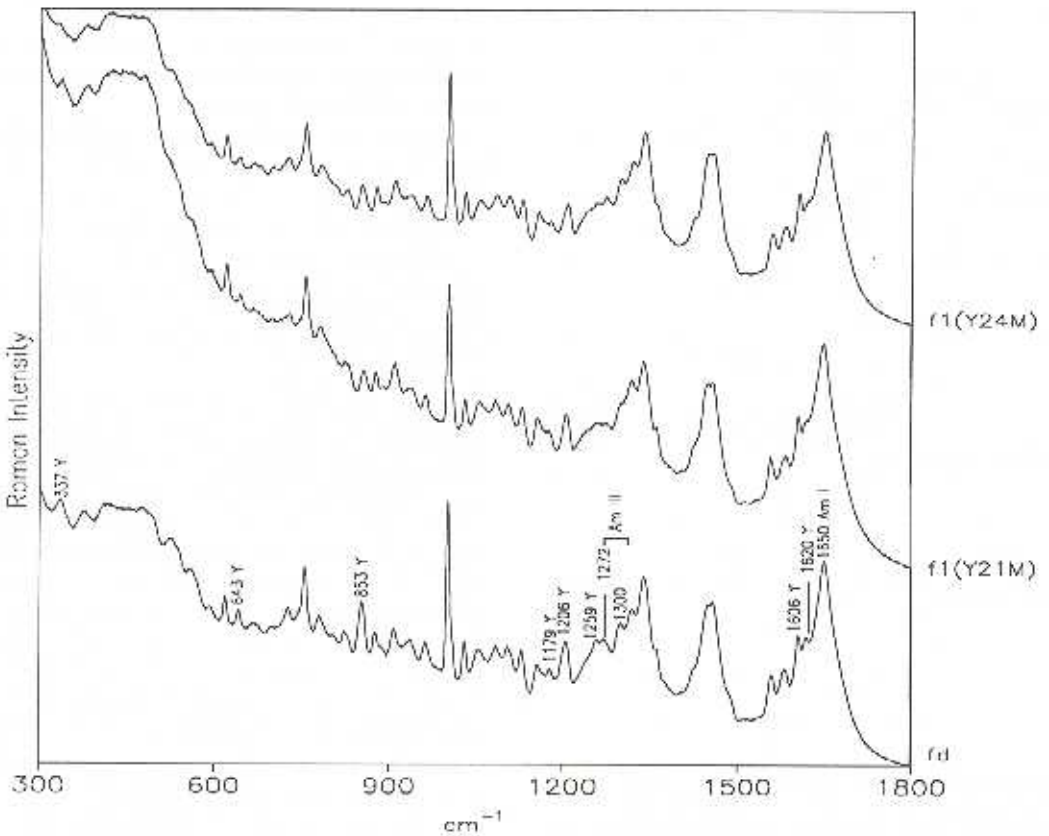

FIGURE 1: Raman spectra in the region 300–1800 cm^{-1} of the wild type *Ff* virus (*fd*, bottom) and single-site mutants, *f1*(Y21M) (middle) and *f1*(Y24M) (top). Spectra were obtained from samples containing virus at 100 $\mu g/\mu L$ in 10 mM Tris at pH 7.8, thermostated at 12 °C. Raman bands due to tyrosine, the intensities of which are affected by the mutations, are labeled. (See text).

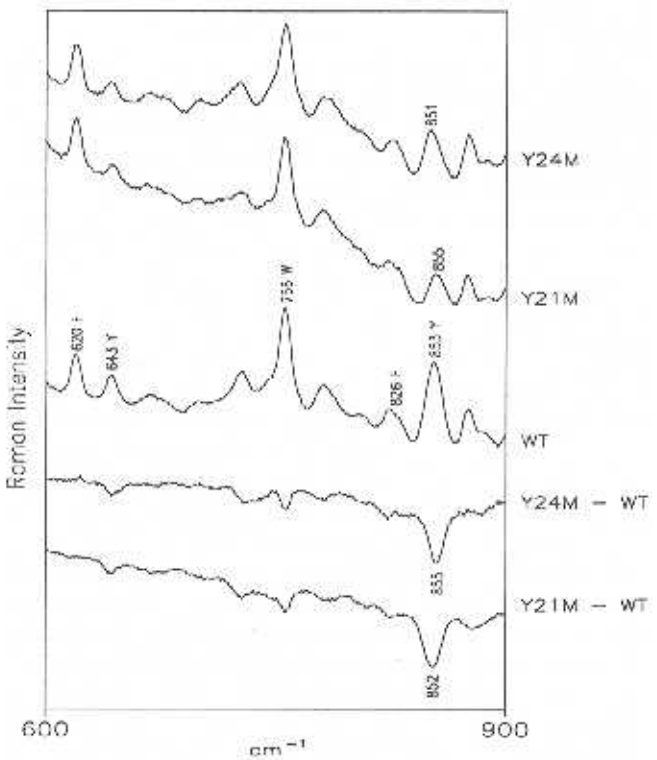

FIGURE 2: Raman spectra in the region 600–900 cm^{-1} of the viruses described in Figure 1 and their corresponding difference spectra, as indicated along the right margin.

the 600–900 cm^{-1} region are discussed in the following section. A detailed analysis of band perturbations occurring outside the 600–900-cm^{-1} interval is given elsewhere (Overman & Thomas, 1994).

DISCUSSION

The Tyrosines (Y21 and Y24) of Ff Lack a Fermi Doublet. Figure 1 shows that the wild-type *Ff* virion (*fd*) and the two single site mutants, *f1*(Y21M) and *f1*(Y24M), exhibit similar Raman spectra throughout the 300–1800 cm^{-1} region. In particular, the spectrum of each virus contains the same amide I (1650 cm^{-1}) and amide III (1270–1300 cm^{-1}) bands, indicating the same subunit α-helical structure (Thomas & Murphy, 1975). Each also displays the same side chain markers for all residues except tyrosine (and methionine). Thus, the bands of the wild type virion at 643, 853, and near 1615 cm^{-1}, which are due virtually exclusively to the two tyrosines (Y21 and Y24), are each diminished in intensity by roughly a factor of 2 as a result of each single site mutation. Additionally, the tyrosines of pVIII contribute substantially to the Raman bands near 1206, 1258, and 1605 cm^{-1}. Each of these bands suffers significant intensity loss with single site mutation. Surprisingly, no intensity change is apparent in the band at 826 cm^{-1} upon mutation of either Y21 or Y24, indicating that this Raman band is probably not due to tyrosine. The assignment of bands at 643, 853, 1206, 1258, 1605, and 1615 cm^{-1} in whole or in part to tyrosine residues is consistent with all available data on tyrosine model compounds (Siamwiza et al., 1975; Takeuchi et al., 1988, 1989). The absence of a tyrosine Raman band near 826 cm^{-1} is unexpected and unprecedented.

The difference spectra of Figure 2 (computed as mutant minus wild type) provide a more detailed perspective of the Raman frequencies and intensities which are altered in the 600–900 cm^{-1} interval upon replacement of either Y21 or Y24 by methionine. [The basis for Raman intensity normalization in Figure 2 is nullification of the 620 cm^{-1} band of phenylalanine. The reliability of this approach for *Ff* has

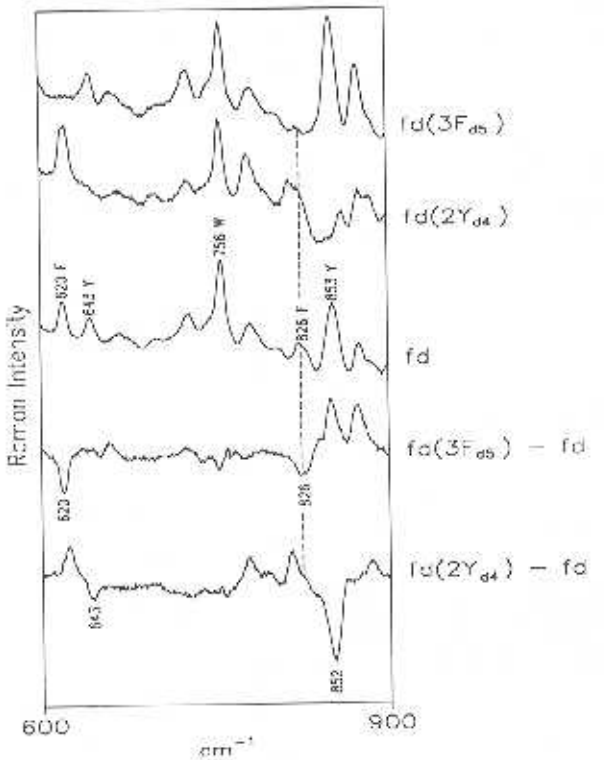

FIGURE 3: Raman spectra in the region 600–900 cm^{-1} of the normal *Ff* virion (*fd*), its deuteriotyrosine [*fd*(2Y$_{d4}$)] and deuteriophenylalanine [*fd*(3F$_{d5}$)] isotopomers, and their corresponding difference spectra, as indicated along the right margin. Phenylalanyl ring deuteration results in virtually complete elimination of the 826 cm^{-1} band (top), confirming its assignment to F and not Y. The enhanced intensity near 815–820 cm^{-1} in the *fd*(2Y$_{d4}$) derivative results from an overlapping band of the deuterated tyrosine ring (Overman & Thomas, 1994), which also appears in the bottom difference spectrum.

been demonstrated (Aubrey & Thomas, 1991).] Figure 2 shows clearly and quantitatively that no significant Raman intensity is lost between 820 and 840 cm^{-1} upon substitution of M for either Y21 or Y24. There are only two possible explanations for this observation: Either the incorporated methionine residue provides fortuitously a Raman band to compensate exactly for any tyrosine band which might have been eliminated or neither Y21 nor Y24 of pVIII contributes a Raman band between 820 and 840 cm^{-1}. The former alternative can be rejected on the grounds that the amino acid methionine exhibits no Raman bands in this spectral region, as verified here (data not shown) and in previous model compound studies (Lord & Yu, 1970). Figure 2 shows that the methionine side chain incorporated into either *f1*(Y21M) or *f1*(Y24M) contributes feebly to the Raman spectrum with only very weak bands near 680, 715, and 790 cm^{-1}, and possibly also near 760 cm^{-1}. *We conclude, therefore, that neither Y21 nor Y24 of pVIII contributes a Raman band at 830 ± 10 cm^{-1} which could be regarded as a typical Fermi doublet component.*

The above conclusion is supported by several additional lines of direct and indirect experimental evidence. First, we show in Figure 3 that incorporation of the ring-deuterated phenylalanyl residue (F-*d5*) in place of normal phenylalanine at the three subunit sites (F11, F42, and F45) is sufficient to eliminate the 826 cm^{-1} band of the normal (unlabeled) virion. Therefore, essentially all of the Raman intensity observed at 826 cm^{-1} in the normal *Ff* virion can be assigned to a phenyl ring mode of phenylalanine, and not to a tyrosine mode. This is extraordinary for a protein Raman spectrum, but is entirely consistent with the Raman spectrum of the free amino acid phenylalanine, which clearly exhibits a weak band near 826 cm^{-1} (Lord & Yu, 1970; Overman & Thomas, 1994). We note further that the 826 cm^{-1} band of phenylalanine is very weak in comparison to the expected contributions from tyrosine in the 800–900 cm^{-1} interval.

Second, the *f1* double mutant, *f1*(Y21F/Y24S), in which both tyrosines are mutated, exhibits a Raman spectral signature in the 600–900 cm^{-1} region which is completely compatible with the foregoing results. The double mutant contributes no bands at 643 and 853 cm^{-1}, but exhibits enhanced intensity at 826 cm^{-1}, due to the additional phenylalanine residue incorporated at position 21 of the pVIII subunit sequence (G. Cesareni and G. J. Thomas, Jr., unpublished results).

Third, we have shown here (Figure 3), as elsewhere (Overman & Thomas, 1994), that incorporation of the phenoxyl ring-deuterated tyrosyl residue (Y-*d4*) in place of normal tyrosine at subunit sites Y21 and Y24 does not alter the 826 cm^{-1} band in the *fd*(2Y$_{d4}$) Raman spectrum, even though all other bands assigned to tyrosine are altered. Further, in Raman spectra of the free amino acid tyrosine and its model compound *p*-cresol, the band near 826 cm^{-1}—a true Fermi doublet component—is clearly shifted by phenoxyl ring deuteration (Takeuchi et al., 1988; Overman & Thomas, 1994). Accordingly, the band of *Ff* at 826 cm^{-1} cannot be assigned logically to tyrosines of the pVIII subunit.

Fourth, thermal and pH denaturations of the *Ff* virion result in a large enhancement of Raman intensity near 825–830 cm^{-1} (Thomas & Day, 1981; Overman & Thomas, 1994). This indicates that a "normal" tyrosine Fermi doublet intensity pattern can be generated for pVIII upon virion disassembly. Evidently, the lower frequency member of the doublet is absent or suppressed in the native virus structure by the tyrosine local environments and/or their interactions.

Fifth, solubilization of the pVIII subunit in detergent micelles or phospholipid vesicles also has the effect of enhancing the Raman intensity near 825–830 cm^{-1} (unpublished results of G. J. Thomas, Jr. and S. J. Opella), consistent with transferring the subunit to a physical state in which the side chain environments of Y21 and Y24 have been altered from and rendered more typical than those existing in the native virus structure. Again, this suggests that the anomalous states of Y21 and Y24 in the virion are determined by the subunit packing arrangement. Interestingly, a similar effect of detergent solubilization on tyrosine marker bands of pVIII has been reported in the ultraviolet resonance Raman (UVRR) spectrum of *fd* excited at 200 nm (Grygon et al., 1988).

Finally, the basic requirement for a Fermi doublet, namely, the accidental degeneracy or near degeneracy of a fundamental and overtone of the same symmetry species (Herzberg, 1945), might not be met for the tyrosines of native *Ff*. We observe (Figure 4) a very weak Raman band at 413 cm^{-1} in normal *Ff*, which is shifted to 387 cm^{-1} in *fd*(2Y$_{d4}$). The frequency of this band is consistent with normal mode Y$_{16a}$, the overtone of which would be a candidate for Fermi resonance with the Y$_1$ fundamental of the phenoxyl ring (Siamwiza et al., 1975). Assuming that the band at 853 ± 2 cm^{-1} in *Ff* (Figure 2) represents the Y$_1$ fundamental in question, and further that the weak 413 cm^{-1} band is Y$_{16a}$, then the overtone and fundamental would be separated by about 27 cm^{-1} (853 − 2 × 413). This is a substantially greater separation of energy levels than projected by Siamwiza et al. (1975) in the model compounds which they examined. These authors generally assumed Y$_1$ ≈ 840 cm^{-1} and observed Y$_{16a}$ ≈ 415–420 cm^{-1}. We speculate that "anomalous" tyrosine ring environments or interactions in the native *Ff* virion could elevate Y$_1$ to ca.

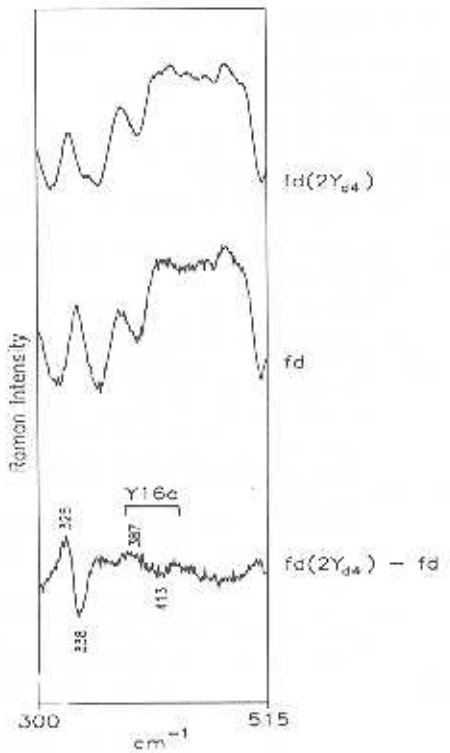

FIGURE 4: Raman spectra in the region 300–515 cm^{-1} of the normal *Ff* virus (*fd*, middle) and its deuteriotyrosine isotopomer [*fd*(2Y$_{d4}$), top], and their difference spectrum (bottom). The proposed Y$_{16a}$ assignment in *fd* and its deuterium-shifted counterpart in *fd*(2Y$_{d4}$) are labeled.

853 cm^{-1}, presumably well above the frequency favorable to Fermi resonance in model compounds and typical proteins, and evidently out of the range required for strong Fermi interaction. Upon filament disassembly or amphipathic solubilization of pVIII (see above), the tyrosine environments could be altered sufficiently to reduce Y$_1$ to a more normal value (ca. 840 cm^{-1}), and Fermi interaction with the overtone of Y$_{16a}$ could become feasible.

Tyrosine Environments in the Protein Coat of Ff Virions. To our knowledge, the absence of a tyrosine marker band near 830 cm^{-1} in the Raman spectrum of a tyrosine-containing protein is unprecedented. In accordance with the well established correlation of Siamwiza et al. (1975), all tyrosine-containing proteins and tyrosyl model compounds heretofore examined have displayed a pair of Raman bands near 850 and 830 cm^{-1} attributable to a tyrosine Fermi doublet. The correlation of Simawiza et al. (1975) for proteins is essentially as follows: (i) When the phenolic OH group acts predominantly as the donor of a strong hydrogen bond to an electronegative acceptor, such as carboxyl oxygen, the intensity ratio of the doublet, I_{850}/I_{830}, achieves its minimum value of 0.30. (ii) When the phenolic OH group acts as both donor and acceptor of moderate hydrogen bonds, for example when exposed to solvent H_2O molecules, $I_{850}/I_{830} = 1.25$. (iii) When the phenolic OH group acts as the acceptor of a strong hydrogen bond from an electropositive donor, such as a lysyl NH_3^+ group, and does not participate in significant hydrogen-bond donation, then I_{850}/I_{830} achieves a presumed maximum value of 2.50. (iv) Finally, when tyrosine is deprotonated at elevated pH to form a phenoxide ion, then $I_{850}/I_{830} \approx 0.7$. For residues Y21 and Y24 in pVIII subunits of the native *Ff* structure, we observe no doublet. This is true of both the wild-type and mutant virus assemblies. We conclude, therefore, that the molecular environments of Y21 and Y24 cannot be rationalized in terms of the correlation of Siamwiza et al. (1975).

To account for the absence of a Fermi component at 830 cm^{-1} in the *Ff* Raman spectrum, we propose that the tyrosine ring environments are not represented accurately by the model compounds examined previously. Two such possibilities are suggested by available experimental data. First, Siamwiza et al. (1975) point out that any factor which lowers the frequency of normal mode Y$_{16a}$ will lead to very high intensity of the Fermi component at 850 cm^{-1} and very low intensity of the component at 830 cm^{-1}, a situation favored by interactions which bleed electron density from the phenoxyl oxygen acceptor. In the extreme case of a phenoxyl oxygen acting as a very strong hydrogen-bond acceptor while the phenoxyl hydrogen is not a donor, removal of negative charge density from the phenoxyl oxygen would be substantial and could lead to an intensity ratio I_{850}/I_{830} exceeding the observed model compound maximum of 2.50. Unfortunately, it is difficult to devise an experimental system to test this hypothesis, and this case was not examined directly by Siamwiza et al. (1975). However, such a situation is compatible with the side-chain environments of Y21 and Y24 in the *Ff* subunit, where proximity to lysyl (K8) and indole (W26) donors could allow such hydrogen bonding (Glucksman et al., 1992; Marvin et al., 1994). Access of hydrophilic groups to the *para*-hydroxyls would also be consistent with reported chemical reactivities of Y21 and Y24 (Marvin, 1990).

A second possibility is a highly hydrophobic aromatic ring environment. We note that Y21 and Y24 are located in hydrophobic segments of the pVIII subunit, which may impose a requirement for hydrophobic packing of this subunit domain in the virion assembly. The location of the aromatic rings of Y21 and Y24 in hydrophobic environments is consistent with a recently proposed structural model for *Ff* which packs Y21, Y24, W26, F11, F42, and F45 of the same and neighboring subunits in an extended hydrophobic cluster (Marvin et al., 1994). The clustering of the aromatic rings of Y21 and Y24 in hydrophobic pockets need not preclude participation of their phenolic substituents in hydrogen bonding interactions, as would in fact be expected on thermodynamic grounds. In support of hydrophobic ring environments for Y21 and Y24 we cite evidence obtained recently from Raman spectra of dilute solutions of *p*-ethylphenol in the hydrophobic solvent, cyclohexane-d_{12} (T. Miura and G. J. Thomas, Jr., unpublished results). The spectra show that in this hydrophobic environment the intensity of the higher frequency Fermi component is much greater than the intensity of the lower frequency component ($I_{850} \gg I_{830}$), i.e., the Fermi doublet intensity ratio greatly exceeds the previously proposed maximum of 2.50. Apparently, the 830 cm^{-1} band becomes feeble as the hydrophobicity of the environment is increased. Interestingly, this physical state was not probed extensively by Siamwiza et al. (1975), although these authors reported one example of a phenol model compound (*p*-cresol) in a non-hydrogen-bonding solvent (cyclohexane) which indeed exhibited a Fermi doublet ratio significantly larger ($I_{850}/I_{830} \approx 5$) than the previously presumed maximum value of 2.50.

The detection of a tyrosine band at 854 cm^{-1}, without a companion near 830 cm^{-1}, in the UVRR spectrum of *fd* (Grygon et al., 1988) is also consistent with our results and structural interpretation. Although the selection rules governing band intensities in off-resonance and resonance Raman spectra are different, the band frequencies are expected to coincide closely. Both the off-resonance Y$_1$ marker reported here (853 ± 1 cm^{-1}) and the corresponding UVRR marker

reported by Grygon et al. (854 cm^{-1}) would appear to support a tyrosine Y_1 fundamental of relatively high frequency in *Ff*, presumably unfavorable to strong Fermi interaction with $2Y_{16a}$.

CONCLUSIONS

The present results show that tyrosine residues in a protein need not give rise to a Raman Fermi doublet between 825 and 855 cm^{-1}, and suggest that phenolic hydrogen bonding interactions are not the sole determinant of protein Raman intensities in this spectral interval. Our findings indicate that structural interpretations of the tyrosine Fermi doublet should be approached with caution in protein Raman spectra when either of the following conditions exists: (1) The protein contains substantially more phenylalanine than tyrosine, resulting in a significant phenylalanine contribution near 826 cm^{-1}, apart from any contributions of tyrosine to the Raman spectrum. (2) The tyrosine is packed within a protein core favoring a strongly hydrophobic environment for the *para*-substituted phenoxyl ring and/or strong hydrogen-bond acceptor interaction of the phenolic oxygen.

The demonstration of a tyrosine singlet, rather than a doublet, is novel and conclusive. Whether the origin of the singlet in *Ff* spectra is determined exclusively by the molecular environments of the tyrosine residues, or by other structural factors as well, remains speculative. A more detailed analysis of environmental and structural factors affecting the tyrosine Fermi doublet is currently in progress in our laboratory.

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