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2 **Raman spectroscopic signatures of echovirus 1 uncoating**

3

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21 **ABSTRACT**

22 During the last decades Raman spectroscopy has entered the biological and medical
23 fields. It enables non-destructive analysis of structural details at molecular level, and
24 has been used to study viruses and their constituents. Here we use Raman
25 spectroscopy to study echovirus 1 (EV1), a small, non-enveloped human pathogen,
26 in two different uncoating states induced by heat treatments. Raman signals of
27 capsid proteins and RNA genome were observed from the intact virus, uncoating
28 intermediate, and disrupted state. Transmission electron microscopy data revealed
29 general structural changes between the studied particles. Compared to the intact
30 virion, the spectral characteristics of the proteins of the heat-treated particles
31 indicated reducing α -helix content with respect to β -sheets and coil structures.
32 Changes observed in tryptophan and tyrosine signals suggest an increasingly
33 hydrophilic environment around these residues. RNA signals revealed a change in
34 the environment of the genome, and in its conformation. The ionized carbonyl
35 vibrations showed small changes between the intact virion and the disrupted
36 particles, which points out to cleavage of salt bridges in the protein structure during
37 the uncoating process. In conclusion, our data reveals distinguishable Raman
38 signatures of the intact, intermediate, and disrupted EV1 particles. These changes
39 indicate structural, chemical and solute-solvent alterations in the genome and in the
40 capsid proteins and lay the essential ground work for investigating the uncoating of
41 EV1 and related viruses in real time.

42

43 **IMPORTANCE**

44 In order to combat against virus infection we need to know the details of virus
45 uncoating. We present here the novel Raman signatures for opened and intact

46 Echovirus 1. This gives hope that the signatures may be used in the near future to
47 evaluate the ambient conditions in endosomes leading to virus uncoating using e.g.
48 CARS imaging. These studies will complement well the structural studies on virus
49 uncoating. In addition, Raman/CARS imaging offers possibility for dynamic live
50 measurements in vitro and in cells which are impossible for example by cryo electron
51 tomography. Furthermore, as viral Raman spectra can be overwhelmed with various
52 contaminants, we find our study being extremely up-to date to demonstrate the
53 importance of the sample preparation for Raman spectroscopy in the field of virology.

54

55 **INTRODUCTION**

56

57 Picornaviruses are small, non-enveloped human pathogens able to cause a wide
58 range of illnesses (1). The subgroup enteroviruses include clinically important
59 echoviruses, coxsackieviruses and poliovirus. These viruses cause a variety of
60 symptoms varying from mild infections to aseptic meningitis, heart muscle damage
61 and paralysis. Enteroviruses have also recently been associated with chronic
62 diseases such as type 1 diabetes, cardiomyopathies and atherosclerosis (2,3).
63 Structural details of these viruses have been obtained by means of x-ray
64 crystallography or at a lower resolution by cryo-electron microscopy (4). Various
65 imaging techniques together with biochemical analysis have revealed important
66 information about their entry to cells and the subsequent events leading to the
67 uncoating and virus replication (5,6). However, information available on the physico-
68 chemical details of genome release events of enteroviral particles is sparse. The
69 uncoating process has been extensively studied by initiating the genome release
70 using heat treatments where intact virions are irreversibly converted to subviral

71 particles that resemble naturally occurring uncoating intermediates (7,8,9). Recently,
72 using such temperature manipulated particles; a long-standing paradigm of the
73 genome release model from the 5-fold axis has been revised (10, 11). Yet, at the
74 present, the biochemical events leading to the genome release as well as the
75 initiating environmental trigger of uncoating is missing (1). Further studies are thus
76 important for addressing one of the events in the enteroviral life cycle, the genome
77 release, and also for the possibility of spotting new potential antiviral targets against
78 related enteroviruses.

79

80 Echovirus 1 (EV1) is a picornavirus belonging to a structurally related group of
81 enteroviruses. Common to all picornaviruses, EV1 is icosahedron-shaped (T=1)
82 assembly of 60 copies of identical protomers comprised of four viral proteins (VP1,
83 VP2, VP3 and VP4), which encapsulate a positive-sense single-stranded RNA
84 genome of approximately 7500 nucleotides. A 14-carbon saturated fatty acid,
85 myristate, is covalently attached to the N-terminus of each picornavirus VP4 capsid
86 protein (12), and it is thought to exit the capsid simultaneously with VP4 during the
87 uncoating process (13). Additionally, studies on poliovirus have shown that the N-
88 terminus of VP1 is externalized to the virion surface during the uncoating process
89 increasing the overall hydrophobicity of the particle (14). Also, the release of the
90 stability-mediating pocket factor, characterized as palmitic acid and located in the
91 core of VP1, is thought to be a prerequisite in the uncoating process, as was shown
92 for bovine enterovirus (15,16).

93

94 Raman spectroscopy provides specific signatures of proteins, nucleic acids and
95 lipids. It can reveal vast amount of information about changes taking place in the

96 chemical content of the particles, in the protein secondary structure and in the
97 physical environment of the particles, which undergo biologically important
98 transformations (17). Raman spectroscopy is a noninvasive characterization
99 technique, which uses visible light laser beams, and thus enables microscopic mode
100 with the same resolution as in fluorescence microscopy. As the acquisition can be
101 fast, processes in real time can be studied. As Raman spectra are only slightly
102 disturbed by water environment, information of molecules in their natural habitat or
103 under a wide range of conditions can be obtained. This makes it an ideal probe to
104 study detailed structural alterations, viral protein assembly, interactions, and
105 dynamics, without any labels or other invasive sample preparation methods.

106

107 Raman spectroscopic studies on viruses, such as Turnip Yellow Mosaic Virus
108 (TYMV) (18), Bean Pod Mottle Virus (BPMV) (19,20), Belladonna Mottle Virus
109 (BDMV) (21) and bacteriophages, such as filamentous bacteriophage Ff (22,23),
110 PRD1 virus (24) and P22 (25-27) have been reported. However, these viruses differ
111 substantially from enteroviruses in several aspects. In the case of plant viruses,
112 TYMV, BPMV and BDMV, the genome is bound to the protein capsid in static form,
113 whereas in the enteroviruses the interior of the virion is spatially disordered with
114 respect to the symmetric protein shell (28). In addition, the subunit packing is
115 somewhat different between these bromoviruses and enteroviruses (28). The
116 bacteriophage PRD1 is icosahedral in shape, but has a lipid envelope. P22 phage is
117 non-enveloped, head-tail structured virus, with 6 prominent tail spikes. All such
118 properties influence the Raman spectroscopic character of these viruses.

119

120 Detailed Raman spectroscopic signatures from the virus uncoating process of

121 enteroviruses, both *in vivo* and *in vitro*, are missing. For such studies linear Raman
122 experiments of the initial and final states are a prerequisite. Here, we characterize
123 vibrational spectroscopic properties of intact EV1 virions and investigate the spectral
124 differences arising in the uncoating process of EV1 particles *in vitro*. We elucidate,
125 first of all, the Raman spectroscopic basis of recognition between the intact virion
126 and the disrupted EV1 particle. Further, we show Raman signature of the uncoating
127 intermediate, which is obtained by heating the intact, infectious particles at 50°C.
128 Previous studies utilizing heat treatments in order to produce and to investigate
129 uncoating intermediates of enteroviruses have produced a wealth of information.
130 Heating enterovirus particles at 50-60°C for varying amount of time has been shown
131 to be able to produce uncoating intermediate (135S), genome ejecting as well as
132 empty (80S) particles indistinguishable from those observed *in vivo* (7,10,11). The
133 Raman spectra of the particles heat-treated at 50°C represent a state where the
134 genome is partially ejected from the capsid, as confirmed by transmission electron
135 microscopy (TEM) and thermal stability assay (29). We conclude that there are clear
136 Raman markers, which address the uncoating stage of the virion. The experiments
137 reported here provide a framework for monitoring the sequence of chemical and
138 conformational changes occurring during enterovirus uncoating in a time-resolved
139 manner.

140

141 **MATERIALS AND METHODS**

142

143 **Cell culture and virus purification**

144 Echovirus 1 (Farouk strain, obtained from ATCC) was propagated in a monolayer of
145 GMK cells and purified using similar overall scheme as described in Ref. (30). To

146 summarize, overnight (24 h) infected cells were collected and repeatedly
147 freeze/thawed (3 cycles) to lyse cellular structures. Bulk cell debris was removed by
148 centrifugation (4500 x g for 15 min). To precipitate protein, 8% (w/v) PEG-6000
149 (Sigma-Aldrich) and 2.2% (w/v) NaCl were added to the supernatant and stirred
150 overnight (24h) at 4°C. Precipitated material was collected by centrifugation (8000 x
151 g for 40 min) and suspended in R-buffer (10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 50
152 mM MgCl₂, 10% (w/v) glycerol). To disrupt membranous structures, 0.3% (w/v) of
153 sodium deoxycholate (Sigma-Aldrich) and 0.6% (v/v) of Nonident™ P 40 substitute
154 (Sigma-Aldrich) were added to the suspension and incubated for 30 min at 4°C. The
155 resulting mixture was clarified by centrifugation (3500 x g for 15 min) and divided
156 equally (<1.5 ml/gradient) on top of 10 ml linear density gradients of 10 to 40% (w/v)
157 sucrose in R-buffer. The gradients were subsequently ultra-centrifuged (86 000 x g
158 for 3 h at 4°C) and fractioned from the top into 500 µl aliquots. Based on optical
159 density measurement at 260 nm (NanoDrop 1000, Thermo scientific), three virion
160 containing fractions (#11, #12 and #13) were pooled and diluted to 10 ml in PBS
161 supplemented with 2 mM MgCl₂. The diluted fractions were dialyzed thrice against 1
162 L of PBS supplemented with 2 mM MgCl₂ using 50 kDa Molecular weight cut-off
163 Spectra/Por® Float-A-Lyzer® Cellulose Ester membrane (Spectrum laboratories).
164 Finally, dialyzed virions were pelleted by ultracentrifugation (93000 x g for 2 h at 4°C)
165 and suspended in a small volume of PBS supplemented with 2 mM MgCl₂. All
166 measured samples were dissolved in the aforementioned PBS buffer. Virions were
167 stored at -80°C.

168

169 **Infectivity of the purified EV1 virions**

170 Infectivity of the purified virus batch was assayed using end-point-titration method to

171 determine 50% Tissue Culture Infective Dose (TCID₅₀). The TCID₅₀ value was
172 calculated as described in Ref. (31) positioning the TCID₅₀ value between the last
173 infected and first non-infected well. Briefly, a monolayer of GMK cells seeded at
174 $5 \times 10^4 \text{ ml}^{-1}$ on a 96-well plate were infected with serially diluted virus stock (starting
175 from 10^{-5} dilution). The progression of the infection was followed daily by light
176 microscope. After 72 h incubation period the cells were stained using crystal violet
177 supplemented with 10% formalin (for 10 min at RT). The detached cells were
178 washed away with water and remaining attached cells were counted as viable and
179 non-infected. For the EV1 batches used in the experiments, the TCID₅₀/ml was found
180 to be 6.1×10^{11} - 1.5×10^{12} .

181

182 **Protein content and composition of purified EV1**

183 Protein content of the purified EV1 was determined using a method described by
184 Porterfield et al. (32) where an average value of VP1-4 molecular mass (23.5 kDa)
185 and an average value of VP1-4 molar absorptivity ($\epsilon_{280} 34440 \text{ M}^{-1} \text{ cm}^{-1}$) were used
186 for the calculations. Protein concentrations of the batches were $0.9 - 3.8 \text{ mg ml}^{-1}$
187 after subtracting scattering component (6.9% of the baseline subtracted absorption
188 signal at 260 nm). The 260/280 ratio was 1.65. Absorption spectrum was recorded
189 with PerkinElmer Lambda 850 spectrophotometer using 10.0 mm optical path quartz
190 cuvette (Hellma Analytics). The protein composition of purified EV1 was analyzed
191 using 12% SDS-PAGE. Sample lane was loaded with $5 \mu\text{g}$ of EV1 and PageRuler™
192 Plus (Thermo Scientific) was used as a molecular weight marker.

193

194 **Thermal stability**

195 Thermal stability of the purified EV1 particles was assayed using method described

196 by Walter et al (29). Fluorescence signal was recorded using Bio-Rad C1000 thermal
197 cycler, and the final sample mixture contained 1 µg of EV1 and 10X concentration of
198 SYBR[®] Green II (Invitrogen). All samples were equilibrated at 20°C for 10 min before
199 starting thermal stability measurements. For the full temperature range scan the
200 fluorescence signal was recorded at 10 s intervals with 0.5°C increments. In
201 additional measurements, the designated temperature was kept constant for 3
202 minutes (50°C) or 10 minutes (60°C), and after heat treatment the sample was
203 cooled back to 20°C at which point the fluorescence reading was taken or loaded to
204 EM grid.

205

206 **Transmission electron microscopy**

207 Morphology of the heated and non-heated virions was visualized by negative
208 staining with 1% (w/v) phosphotungstic acid (Sigma-Aldrich). The formvar-coated EM
209 grids were glow-discharged (EMS/SC7620 Mini Sputter Coater) and samples were
210 deposited on the grids for 15 seconds after which the excess sample was blotted
211 away (Whatman 3MM). Next, negative stain was added for 1 minute and removed as
212 before. Samples dried for at least overnight were imaged with JEM-1400 (JEOL)
213 transmission electron microscope with 50 000 x magnification (80 kV).

214

215 **Raman spectroscopy**

216 For all Raman viral experiments, aliquots of 6-10 µl with sample concentration 0.9
217 mg ml⁻¹ for dried samples and 3.8 mg ml⁻¹ for aqueous samples were deposited
218 either on a CaF₂-window, or sealed in a glass capillary (American Dade SMI
219 Capillaries: P5070-902). Heating of the aqueous samples was performed in a water
220 bath, either at 50°C for 3 min or at 60°C for 10 min. The dried sample was allowed to

221 dry for 15 min at ambient temperature. The Raman spectra were measured with a
222 home built Raman setup in a backscattering geometry. In this setup a laser beam of
223 solid-state laser (CNI) with excitation wavelength at 532 nm was focused onto the
224 sample with a spot diameter of 3 μm using a microscope objective (Zeiss, Plan-
225 NEOFLUAR 10x/0.30). Raman scattering was recorded using a CCD camera
226 (Andor, Newton DU940P-BV) attached to an imaging spectrograph (Acton Spectra
227 Pro 2500i) with entrance slit of 50 μm and grating of 600 grooves/mm. Raman
228 spectra were accumulated using 30 x 10 s exposures. Laser power of 45 mW for
229 dried and 200 mW for aqueous samples was used. From each scan a dark spectrum
230 was subtracted. The reference spectra of water, buffer solution, empty capillary, and
231 CaF_2 -window were detected with the same settings and subtracted from the sample
232 spectra. The fluorescence background was defined by fitting a polynomial curve to
233 the measured spectrum from the points where no Raman signal is expected to be
234 present, and subtracted from the data. After this procedure, no other spectral
235 signatures than viral constituents were obtained.

236

237 Spectra of the aqueous samples of concentration 3.8 mg ml^{-1} represent mean values
238 of measurements from three different EV1 batches normalized to the Phe peak at
239 1003 cm^{-1} with a floating average with a span of three data points (2.7 cm^{-1}). In
240 addition, the differences between room temperature and heated samples were
241 analyzed in four separated spectral sections, $610\text{-}945 \text{ cm}^{-1}$, $945\text{-}1145 \text{ cm}^{-1}$, 1145-
242 1520 cm^{-1} , and $1520\text{-}1800 \text{ cm}^{-1}$, which were normalized to the mean signals of each
243 section and smoothed with a floating average with a span of seven data points (10
244 cm^{-1}).

245

246 **Radial distribution and positioning of salt bridges in EV1**

247 The atom coordinates were gathered from EV1 protomer (PDB entry: 1EV1) using
248 UCFS Chimera (33) build 1.8. Salt bridges were identified manually scoring only
249 arginine and lysine as salt bridge forming residues with carboxylic oxygen of Glu and
250 Asp. In case of many possible connections ($<3.5 \text{ \AA}$) the closest was chosen resulting
251 in one connection per residue.

252

253 **RESULTS**

254

255 **Sample purity and heat-induced uncoating of EV1**

256 The Raman analysis of viral particles imposes a demand for highly homogenous and
257 pure samples. The sucrose gradient purification scheme used here resulted in a
258 single clearly visible band. The fractionated gradient showed a local absorbance
259 maximum at 260 nm in fraction 12, while five top fractions also showed high optical
260 density; no visible banding was observed (Fig. 1A). After dialysis the collected
261 fractions (Fr#11-13) were analyzed for protein composition using 12% SDS-PAGE.
262 Fig. 1C shows that the purified EV1 was contaminant-free in the molecular weight
263 range of 250-10 kDa, while the prominent capsid proteins, VP1-3, banded next to 35-
264 25 kDa markers, as expected. To quantify the protein concentration of a virus
265 sample, a UV-Vis spectroscopic method introduced by Porterfield et al. (32) was
266 used. In our hands, the above-mentioned spectroscopic approach has been found to
267 be in a reasonable agreement with the more traditional Bradford assay (data not
268 shown). The method incorporates scattering correction in the resulting absorption
269 spectrum, with 6.9% of the total signal. This subsequently affects the 260/280 ratio,
270 which was 1.65 for dialyzed particles (Fig. 1B). In literature, the reported 260/280

271 ratios for enteroviruses vary considerably (34-36), partially due to differences in
272 genome contents, but also resulting from uncorrected spectra. However, as the
273 genome to protein ratio (260/280) does not reflect virion integrity, EV1 was visualized
274 by TEM and was found to consist of a highly homogenous population of intact
275 particles approximately 25 nm in diameter (Fig. 1D).

276

277 Since the EV1 capsid proved to be non-permeable to the SYBR Green II dye (Fig.
278 2A), thermal stability assay was used along with TEM to evaluate the extent of
279 genome egress from intact EV1 particles. Fig. 2A indicates the melting temperature
280 (T_m) for intact EV1 particles to be 51°C. Noting that the increase in SYBR Green II
281 signal begins to rise around 40°C and accumulates approximately 3 minutes of
282 effective heating time prior to the 51°C T_m mark, 3 minute heating at 50°C was used
283 to produce the so-called uncoating intermediate particles (Fig. 2B). The population of
284 these genome-releasing virions was visualized by TEM (Fig. 3). After 3 minutes of
285 heating at 50°C great majority of the visualized particles were connected to a
286 protruding density (Fig. 3B). Additionally, Figs. 3A and 3B show both the intact (20°C)
287 virions and partially disrupted virions (50°C) to be structurally organized and
288 symmetrical in shape. Similar particles have been obtained earlier using polioviruses
289 (11). Heating the intact EV1 virions at 60°C resulted in a complete particle disruption
290 (Fig. 3C). Over all, homogeneously genome deprived but structurally intact EV1
291 particles were challenging to produce by heat treatment alone (data not shown). The
292 heat-induced chemical and structural alterations in the particles treated at 20°C,
293 50°C and 60°C temperatures were then further probed by Raman spectroscopy.

294

295 **Raman analysis of viral particles**

296 The Raman spectrum of the intact virion (Fig. 4) shows a large amount of vibrations
297 that can be assigned reasonably well using literature values as expressed in Tables
298 1A and 1B, for proteins and RNA, respectively (17,24,37,38,39). The most prominent
299 vibrational region is the aliphatic C-H stretching complex shown at around 2900
300 cm^{-1} . Here we concentrate, however, to the fingerprint region from 600 to 1800 cm^{-1} ,
301 where most previous biological applications of Raman spectroscopy have been
302 performed, as it has higher sensitivity to structural and chemical nature of particles
303 (see e.g. 18,23). The best known spectral features found at typical positions (17) in
304 the Raman spectra of the virus are Am I and Am III bands at around 1670 cm^{-1} and
305 in the region between 1230 and 1300 cm^{-1} , respectively. The anti-symmetric C-C
306 stretching modes of aromatic amino acids (Phe, Tyr, and Trp) locate in the region of
307 1580-1615 cm^{-1} and the ring breathing modes of aromatic amino acids at around
308 1000 cm^{-1} . Other side-chain vibrations emerge at around 600-900 cm^{-1} , and the
309 methyl/methylene vibration bands locate at around 1350 and 1450 cm^{-1} . Many RNA
310 vibrations partially overlap with the protein bands, although a large amount of
311 vibrations can still be specified. The ring breathing modes of RNA bases appear in
312 the region below 800 cm^{-1} , ribose and phosphate signals between 800 and 1100 cm^{-1} ,
313 and the stretching vibrations of the bases at higher wavenumbers (1200-1600 cm^{-1})
314 (39,40).

315

316 Raman spectra of viral particles in buffer environment show clear similarities with
317 spectra of the dried particles (Fig. 4B) and basically all spectral features can be
318 observed from the solution with concentration as low as 0.9 mg ml^{-1} . The details of
319 the spectra are, however, more clearly distinguished from the noise-level of our
320 experiment at virus concentration of 3.8 mg ml^{-1} , which is, thus, used in the

321 characterization of the heat-induced changes of the viral particles.

322

323 By detecting Raman spectra of the particles treated with different temperatures we
324 aimed to indicate chemical and structural changes between the intact virus particles
325 and an uncoating intermediate virion particle, as well as with an end-point structure
326 where the virion particle was disrupted by heat treatment (morphology of particles
327 obtained with TEM (vide supra, Fig. 3)). As shown in Fig. 5, the Raman spectra of
328 the particles heat-treated at 50°C and 60°C deviate clearly from that of the intact
329 virion. As the heat treatment changes the form (Fig. 3) and possibly also the solvent
330 shell of the particles, the scattering and fluorescence properties of the particles differ.
331 This creates challenges in baseline determination and normalization of the spectra,
332 and due to this the Raman spectra were dissected in four individual spectral regions
333 from 610 to 1800 cm^{-1} . Figs. 5A-D show the differences between the particles. In
334 most cases, a gradual change of Raman spectra from intact to 50°C treated, and
335 then to 60°C treated particles, is observed. This effect can be expected according to
336 the TEM analysis as well (Fig. 3), which shows basically a full disruption of the
337 particles after 60°C heat treatment.

338

339 The largest changes in the spectra are observed in signals assigned to the Amide
340 bands, methyl/methylene signal at 1446 cm^{-1} , and RNA signals at 750-800 cm^{-1} ,
341 although many changes locate in regions where multiple factors affect to the spectra.
342 Interestingly, the 1446 cm^{-1} band, reflecting C-H vibrations from the proteins, is more
343 affected than the corresponding band for RNA molecules at 1478 cm^{-1} . Thus, the
344 ratio between 1446 and 1478 cm^{-1} conveniently indicates changes of signals
345 between proteins and genome, respectively, in the viral particles. The Am I and Am

346 III regions, shown in Fig. 5, reveal information on the changes in the secondary
347 structure of the coat proteins in the heat-treated particles. The differences between
348 the spectra are the most pronounced at 1630-1650 cm^{-1} and 1670 cm^{-1} (Fig. 5D).
349 Broadening of the Am I peak is largest in the spectrum of the intact virus. On the
350 lower energy side of Am I vibration, the 1575 cm^{-1} band, which originates from ring
351 breathing modes, both from Trp as well as from adenine and guanine of RNA
352 (39,41), shows higher intensity in the heated particles, especially in the spectra of
353 the particles treated at 60°C.

354

355 Most of the heat-induced changes grow gradually as the heat-treatment becomes
356 more intensive. Some of the changes, however, are only evident after heat treatment
357 at 50°C, namely, the decrease of signals at 990 and 1650 cm^{-1} . These changes
358 locate at sites where both RNA and protein vibrations attribute to the signals. At
359 around 990 cm^{-1} , there are RNA vibrations from ribose phosphate (39), and protein
360 derived signals from proline, arginine and tyrosine residues, as well as the nearby
361 phenyl alanine signal at 1003 cm^{-1} (37). At 1650 cm^{-1} the signal originates from the
362 amide bonds of proteins (17).

363

364 Apart from major differences in the Raman signals, subtle signal variations along the
365 spectra can reveal information from important details of the EV1 uncoating process.
366 These changes along with the assignments are indicated in Tables 1A and 1B. For
367 example, a slight change in the signal at around 1710 cm^{-1} in the spectra of the
368 particles treated at 50°C can be observed (Fig. 5D). This signal increase could imply
369 protonation changes of carboxylic groups of Glu residues (42). In ideal case, such
370 increase of signal of the protonated forms of Glu should lead to decrease of signals

371 of COO⁻ population, found from region between 1390 cm⁻¹ and 1410 cm⁻¹, which,
372 however, is hard to detect in Fig. 5C from spectrum of viruses heat-treated at 50°C.
373 Another small change, a shift to lower wavenumbers, or decrease of signal at the
374 higher frequency side of the band, is seen in the peak at 1122-1128 cm⁻¹ (Fig. 5B).
375 This peak represents non-aromatic amino acid residues, but it might also include
376 some fatty acid vibrations. There are two kinds of fatty acids in EV1 capsid, myristate
377 and palmitic acids, the first covalently attached to VP4 and the second buried in VP1
378 formed pocket. No obvious fatty acid peaks can be detected from the spectra, but
379 there are small decreases in signals of heat-treated viruses at the fatty acid signal
380 sites at 1063 cm⁻¹, 1129 cm⁻¹, and 1296 cm⁻¹ (37), that could be consistent with heat-
381 induced changes in the local environment of the fatty acid molecules, or reduction of
382 fatty acid molecule amounts at the measured spot of the sample.

383

384 The Fermi doublets of the aromatic amino acids Trp and Tyr are often used as
385 indicators of indole environment hydrophobicity and phenolic hydrogen bonding.
386 Neither Fermi doublet stands out very clearly from the spectra, but can be assigned
387 to specific signals none the less. Small changes are seen in signals assigned for the
388 Tyr Fermi doublet at 829 and 854 cm⁻¹ (Fig. 5A), where heat treatments reduce the
389 ratio I_{829}/I_{854} , indicative of a greater hydrogen bond acceptor role, or exposure to the
390 solvent, of the average phenoxy group after heat treatments (43). The Trp Fermi
391 doublet at 1340 and 1360 cm⁻¹ shows a small increase in the signal at 1340 cm⁻¹
392 after heat treatment (Fig. 5C), indicating an increasingly hydrophilic environment of
393 Trp residues (44,45), although the overlapping RNA signal assigned to the larger
394 peak at 1335 cm⁻¹ might affect this as well. Trp band at around 880 cm⁻¹ has been
395 considered a marker for Trp hydrogen bonding, with signals at 882-883 cm⁻¹

396 indicating Trp residues with no hydrogen bonding, and at 871 cm^{-1} with strong
397 hydrogen bonding (44). In intact viruses this Trp signal is a single peak centered at
398 877 cm^{-1} , and after heat treatments the signal divides to two peaks at 873 and 881
399 cm^{-1} , indicating changes in Trp hydrogen bonding status. The frequency of the Trp
400 signal near 1550 cm^{-1} has been reported to be indicative of changes in side chain
401 conformation (46-48), and there are heat-induced changes in this region in our data
402 (Fig. 5D). Particularly, in the spectrum of the intact virus this signal overlaps with the
403 peak at 1572 cm^{-1} , and after heat treatment there appears a new peak slightly under
404 1550 cm^{-1} , suggesting a considerable change in conformation of some of the Trp
405 residues. Similar change has been noticed with Bovine enterovirus uncoating with
406 resonance Raman spectroscopy (46). However, also RNA signal appears relatively
407 close at around 1578 cm^{-1} . Yet another Trp peak, at 756 cm^{-1} , also shows apparent
408 heat-induced changes, complementing the interpretation that Trp residues undergo
409 significant changes upon virus uncoating.

410

411 Partially overlapping with the Trp 756 cm^{-1} signal is an RNA signal at 783 cm^{-1} ,
412 coming from the C/U ring breathing modes (Fig. 5A). This signal has been reported
413 to change in intensity due to changes in the environment, particularly in non-helical
414 regions of RNA (39,49). In our experiment, there are heat-induced changes around
415 this peak. A small increase is also seen in the symmetric phosphate stretching
416 vibration signal at 811 cm^{-1} , considered a marker for A form RNA helix, suggesting a
417 change in RNA backbone conformation. Most of the RNA signals somewhat overlap
418 with protein signals - however, RNA can be considered to be the main source of the
419 peaks at 1335 and 1478 cm^{-1} . More detailed assignments of signals to RNA
420 vibrations are expressed in Table 1B (39). Most RNA assigned signals above 1200

421 cm^{-1} slightly increase in intensity after heat-treatments, with one exception. The
422 decrease at 1300 cm^{-1} could come from changes in cytosine or adenine ring
423 stretching vibrations, although it could also be due to fatty acids or changes in
424 protein secondary structures. A signal just below 1050 cm^{-1} , that can be assigned to
425 RNA P-O and sugar phosphate -C-O- stretching, only appears after heat-treatments.

426

427 **DISCUSSION**

428

429 Virus particles of various species or in different assembling or uncoating states
430 deviate from each other in terms of structure and chemical content. In this study,
431 EV1 viral particles, in three different states, are characterized both in terms of
432 structural and chemical information. The intact virion, the uncoating intermediate
433 produced after heating the virion at 50°C for 3 min, and the disrupted viral particle
434 treated at 60°C for 10 min, all showed different types of structures obtained by TEM
435 analysis. Also, the Raman spectroscopy of different states produced unique spectral
436 features.

437

438 The Raman spectrum of a complex biological sample is a mixture of spectral
439 contributions from all Raman active molecular groups contained in the sample, and
440 therefore it enables studying simultaneously all sections taking part in the biological
441 processes and ultimately determining the sequence of the changes taking place in
442 the molecule. As a result, the Raman spectrum of a virus consists of numerous,
443 overlapping bands and peak shoulders. The most prominent spectral features, seen
444 in the spectra of large polypeptides, are Am I originating from the polypeptide chain
445 C=O stretching, and Am III, mainly resulting from the coupled C-N and N-H bending

446 motions. These are widely used as indicators of the secondary structure of proteins
447 (17). Other informative Raman marker bands originate from the aromatic amino
448 acids. Although Raman spectra of biological material are usually interpreted mainly
449 as indicators of secondary structure conformation, other factors in the local
450 environment influence strongly to the position, size, and shape of the Raman signal.
451 Local electric fields, polarity and protonation state of the vibrators (50) play an
452 important role, which means that Raman signals are sensitive to ions, charged
453 amino acids, and even charged phosphates in the protein or in the genome. As
454 shown in this and other studies (18-20,22-27), Raman spectroscopy can provide vast
455 amount of information about the character and status of virion particles. In many
456 cases, however, all information is difficult to distil out from a single set of data. In the
457 following we will condense the essential features of the Raman fingerprint of EV1
458 and discuss some of the crucial factors needed to consider when striving for the
459 genuine Raman spectra from viral samples in aqueous environment.

460

461 As Raman signals are relatively weak, in comparison with for example fluorescence
462 signals of fluorophores, relatively high sample concentrations are needed. Typically,
463 the reported Raman spectra of viral particles are measured with concentrations up to
464 80-100 mg ml⁻¹ (18-27). Low sample concentrations are favorable when aiming to
465 measure the viral particles in non-aggregated form. On many occasions the
466 scattering intensity of viruses is small, and the changes at low concentrations are
467 hidden inside the overruling background spectra. This leads to challenges in
468 detecting the Raman signal of the sample and accurately subtracting the solvent and
469 other background from the weak viral spectra (41). It is also important to keep in
470 mind that some buffers include groups that have similar vibrations as many biological

471 samples, or in the worst case are quite reactive (51), and therefore can – if not
472 obscure the spectra, at least skew the interpretation. Other informative vibrations
473 may be deluged in a heterogeneous sample containing leftovers, such as sugars,
474 from the purification procedure. The material besides the sample may then result in
475 the biggest changes in the Raman spectra, overlapping the small changes taking
476 place in the actual sample. Alternatively they may influence the behavior of the
477 sample, for example sucrose is known to stabilize protein structure (52). In our
478 measurements, the viral particles were clearly dominated by sucrose contamination
479 even after dialysis with 2 kDa molecular weight cut off (data not shown), and were
480 properly purified only with higher molecular weight cut off of 50 kDa. Also, in a
481 recently published article of virus-like particles, there are clear - yet identified -
482 sucrose leftovers in the Raman spectra (53). It is important to notice that because
483 Raman spectroscopy “sees everything”, the purity and homogeneity of the samples
484 is crucial. Even smallest changes in the purification method used can cause
485 additions and interference to the spectra, which can lead to erroneous interpretation.
486 This means the purification procedure must be paid critical attention to, and, as a
487 result, better quality spectra containing more information are achieved. Further, once
488 a spectrum of a pure viral sample has been obtained, Raman spectroscopy could be
489 used as a very sensitive probe for impurity studies for analytical purposes.

490

491 To the best of our knowledge, there are no previous Raman studies on EV1 and its
492 close relatives. Bovine enterovirus and rhinovirus have been studied by means of
493 surface enhanced Raman scattering (SERS) and ultraviolet resonance Raman
494 spectroscopy (54,55). Notable is that, in the case of SERS, the choice of the
495 substrate material, and in the case of resonance Raman studies, the choice of the

496 laser wavelength, all contribute to the intensity of each Raman transition. Our
497 spectra of EV1 are similar especially to those obtained for the BPMV virion (20),
498 which shares considerable structural similarity with picornaviruses (56). Li et al also
499 observed differences in the Raman spectra between crystalline and liquid BPMV
500 viral particles, and concluded changes mainly originating from the packed RNA
501 molecules having different electrostatic environments in the samples (57). It should
502 be recognized, though, that one particular difference with most of the previously
503 reported linear Raman data is that our Raman spectra have been measured with
504 much lower protein concentrations, in our study being under 4 mg ml^{-1} versus the 80
505 mg ml^{-1} or more in other studies (see e.g. 19,20,24). The amount of aggregation and
506 interaction between viral particles at such concentrations could diminish the
507 differences compared to the crystalline state. In our EV1 samples there are
508 differences between spectra measured in solvated and dried states, for example a
509 broadening or a shift in Am I band to higher wavenumbers, indicating a higher
510 amount of disordered secondary structures in the dried sample. On the other hand,
511 the width of some bands originating from individual amino acids, like the Phe band at
512 1003 cm^{-1} , show similar width in spectra of dried and solvated samples. Drying
513 removes the stabilizing hydration shell (58) and can influence the stability, cause
514 aggregation, and hamper at least certain parts of capsid structures. This also implies
515 that the SERS technique (55), where viral particles are attached to metallic surface
516 or nanoparticles, can reveal somewhat different vibrational spectroscopic information
517 than when measuring viral particles normally diffusing freely in a buffer solution.

518

519 Care was taken to remove contribution of the wagging mode of water molecules from
520 the Raman data of the diluted samples, which has been addressed to be essential

521 for a proper analysis of the Am I modes (41). The Raman Am I band centered at
522 1668 cm^{-1} indicates β -sheets as the predominant secondary structure of the capsid,
523 which is consistent with the known structure of the major capsid proteins, VP1, VP2
524 and VP3 in EV1 (59). Temperature induced alterations around 1653 cm^{-1} reveal,
525 however, a decrease in α -helical secondary structure due to the partial unfolding of
526 the shell subunits especially at the intermediate state. The decreasing signal in Am
527 III area at around 1300 cm^{-1} could also be due to reduced α -helical content of
528 proteins. This indicates that the heat-induced uncoating is dominating in the α -helical
529 regions of the protein. The signals assigned to β -sheets and coiled structures in Am I
530 and III regions, at 1668 and 1240 cm^{-1} , respectively, are at the same time increasing
531 in magnitude, suggesting that some of the α -helix structures are converted to β -
532 sheets or coils. Flexible loops between structured domains are known to make
533 protein dynamics less constrained, and serve a critical role in the uncoating process.
534 For example, these mechanically uncoupled structures could enable the capsid
535 expansion, which is clearly visible from the TEM-image of the uncoated EV1 particle
536 (Fig. 3C).

537

538 Signals of aromatic amino acids reveal information on hydrogen bonding,
539 hydrophobicity, and side chain conformation of these residues (43-48). Many of
540 these informative signals of EV1 change with heat treatments. Small changes are
541 seen in the Fermi doublets of Tyr and Trp that change with hydrogen bonding of Tyr
542 and hydrophobicity of Trp environment. Changes in the 877 cm^{-1} signal indicate
543 variation in Trp hydrogen bonding status, and the appearance of a new Trp signal at
544 around 1550 cm^{-1} indicates changes in side chain conformations. All these signals
545 point to the same direction as the changes seen in secondary structures of viral

546 proteins and TEM-images, i.e. loosening of the capsid structure and subsequent
547 exposure of these residues to water.

548

549 Obtaining Glu vibrations of protonated carboxyl group suggests protonation
550 processes to take place in the heat-induced uncoating of EV1. In the unprotonated
551 state, the negatively charged Glu may interact with positively charged residues (e.g.
552 Lys, Arg) to form salt bridges, which have been shown to be important stabilizers of
553 HIV, influenza virus, filamentous viruses, and Bacteriophage hk97 (60-63). These
554 salt bridges may be involved in constrained network of interactions within one
555 subunit or between two neighboring units, clamping the domains in a considerably
556 more stable capsid structure. Our data suggest that salt bridges may be involved in
557 fine-tuning the metastable capsid structure of EV1. By studying the EV1 structure,
558 the distribution of possible salt bridges in the EV1 structure were shown to reside in
559 the interior of the capsid or buried within the capsid structure (Figs. 6A,B).
560 Interestingly, the enterovirus uncoating intermediates reported are shown to have
561 lost the internal protein VP4 (13), which mediates two inter-protomer and one inter-
562 VP salt bridge connection (Fig. 6C). It is thus tempting to speculate that, at least
563 partially, the extraction of VP4 from the capsid particle might be controlled by salt
564 bridges. In comparison to the native virion, the heat-induced uncoating intermediate
565 of EV1 showed a small increase in the protonated carboxylic acid stretching. Also, a
566 small decrease in signals that could be assigned to fatty acids was detected. The
567 disconnected salt bridges, together with the expelled fatty acid chains, are expected
568 to shift the capsid dynamics into a more flexible motion as these constrains are
569 removed.

570

571 In addition to the changes in the capsid proteins, several heat-induced changes in
572 the RNA signals were observed. In general, the changes are difficult to interpret as
573 particular structural changes, but more like a general change of the solute-solvent
574 interactions of the RNA molecule. Especially, the signal variation at 783 cm^{-1}
575 suggests that there are changes in the RNA solvation shell (39,49). Both the ejection
576 of genome and an increased permeability of the capsid structure could explain these
577 changes in the signal sensitive to the environment of RNA. Still, also a small
578 increase in the A form helix marker band at 811 cm^{-1} (39,40) after heat treatment at
579 60°C suggests that RNA is no longer bound by the intact capsid structure and refolds
580 to the A form double helical structure.

581

582 In conclusion, we were able to acquire signature Raman spectra that distinguish
583 between the intact and uncoated EV1 diluted in buffer at relatively low
584 concentrations. Very little vibrational spectroscopic data exists of the conformational
585 changes during the viral uncoating so far. The observed Raman signatures are in
586 good agreement with the present knowledge of enteroviral uncoating. For example,
587 the UV-resonance Raman study comparing the intact and empty bovine enterovirus
588 particles (46) revealed increasing hydrophobicity of the virus, or moving of Trp
589 residues to increasingly hydrophilic environment during the viral opening process,
590 consistent with our measurements. However, the detected differences between the
591 intact virion in dried phase and in the liquid state indicate that the natural
592 environment is essential in order to retain all the information from the uncoating
593 process. Most biological macromolecules are physiologically active in aqueous
594 solutions, and water molecules are supposed to play a crucial role in the function
595 and structure of biomolecules.

596

597 The Raman signatures of EV1 virion particles consist of numerous signals, the most
598 pronounced features being 1) Amide bands: The Am I became less broad after the
599 uncoating process and revealed a decrease of α -helical structures and an increase
600 of irregular structures or β -sheets, 2) Aromatic amino acids: The vibration modes of
601 tryptophan and tyrosine residues suggest loosening of the capsid structure with
602 increasing hydrophilicity around these residues, 3) Chemical changes in the virion
603 particles: The carbonyl vibrations showed small changes between the native virion
604 and the heated particles, which indicates at least partial disruption of salt bridges, 4)
605 Genome signals: RNA signatures indicate both changes in the environment of the
606 genome, and a change in RNA conformation.

607

608 The observed differences in the Raman spectra between the intact and uncoated
609 virions give novel insight into the structural changes occurring during virus opening.
610 Most probably the findings are similar for the enteroviruses that are close relatives to
611 EV1. The prominent Raman marker bands of the intact virion, intermediate uncoating
612 state of the virion, and disrupted virion particle represented here also enable *in vivo*
613 studies of factors leading to viral uncoating in cellular structures with Raman
614 mapping and coherent anti-Stokes Raman Scattering microscopy.

615

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617

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624

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811

812 Table 1. A collection of obtained frequencies of the vibrations, together with their
 813 assignments, based on previous studies (17,24,37,38,39).

814

815

TABLE 1A A collection of obtained frequencies of the vibrations of proteins found in this study. The assignment is based on the previous studies (17,24,37,38).

Assignment	Dried sample, frequency, cm ⁻¹	Aqueous sample, frequency, cm ⁻¹	Heat-induced effect
Phe	623	620	+
Tyr	644	642	
C-S	670	668	
Trp	758	756	+
Tyr	826,855	829,854	-(829)/+(854)
Trp	887	873-883	- (60°C)
AmIII,	960-978	952-977	
Phe	1003	1003	
Phe	1031	1031	
CC, CH	1071-1096	1073-1098	+
non-aromatic side chains	1127	1122-1128	+(1120)/-(1130)
CH ₃	1156-1176	1160-1178	
Phe, Tyr	1208	1205-1210	
Am III	1230-1300	1230-1300	+(1240)/-(1300)
Trp, CH ₃	1318-1378	1322-1383	
Asp, Glu -COO ⁻	1390-1404	1390-1402	+(60°C)
CH ₂	1446-1458	1446-1458	-
Trp	1555,1573	1549,1572	+
Phe		1588-1594	-
Phe, Trp	1605	1603	
Tyr, Trp	1613,1618	1612,1616	
Am I	1669	1668	+
Asp, Glu -COOH		1710	+(50°C)
Abbreviations: Am, amide.			

816

817

818

TABLE 1B A collection of obtained frequencies assigned to the RNA molecule from the intact virus particles. The assignment is based on the study of Hobro et al (39).

Assignment	Dried sample, frequency, cm ⁻¹	Aqueous sample, frequency, cm ⁻¹	Heat-induced effect
A ring stretching (726)	726	722-727	
C/U breathing/stretching (787)	783	783	changes
O-P-O symmetric stretching (813)	811	811	+ (60°C)
Ribose-Phosphate,-C-O-stretching (919)		914-921	-
Ribose-Phosphate,-C-O-stretching (977)		969-980	-
P-O stretch, sugar phosphate –C-O- stretching (1047)	1044	1044sh	+
PO ₂ ⁻ symmetric stretch (1097)	1091-1100	1090-1100	
U/C ring stretching (1253)	1252	1250	
C/A ring stretching (1300)	1300	1300	-
A,G,U (1336)	1336	1335	+
U/C ring stretching (1460)	1460	1458	-
A/G ring stretching (1485)	1480	1478	+
A/G ring stretching (1578)	1576	1578	+

819

820

821

822 **FIGURE LEGENDS**

823

824 **FIG. 1.** The scheme used to purify EV1 produces homogenous population of intact
825 virions. (A) Sucrose gradient fractionated from the top shows clear separation in
826 optical density (OD₂₆₀) between bulk, low-density material, and EV1 virions.
827 Fractions 11, 12 and 13 were collected for further purification. (B) Absorption
828 spectrum of Dialyzed EV1 (dashed line) and light scattering corrected spectrum
829 (solid line) normalized at 260 nm for better comparison between the two. The

830 scattering component was calculated to be 6.9% of the total signal and 260/280 ratio
831 was 1.65. (C) SDS-PAGE analysis of the purified EV1 samples shows no protein
832 contaminants in the range of 250-10 kDa, while prominent viral proteins VP1-3 band
833 between 35 kDa and 25 kDa markers. (D) EV1 particles contrasted by negative
834 staining and visualized by TEM show homogenous population of intact virions
835 approx 25 nm in diameter. Scale bar is 100 nm.

836

837 **FIG. 2.** Thermal stability analysis of intact EV1 virions. (A) Fluorescence traces of
838 SYBR Green II in the presence of EV1 show steep increase starting from around
839 40°C and a midpoint at 51°C (solid line). The SYBR Green II by itself shows no
840 fluorescence increase as a function of temperature (dashed line). The fluorescence
841 is normalized with respect to the initial fluorescence. (B) EV1 was incubated with
842 SYBR Green II at 50°C and 60°C for 3 and 10 minutes, respectively, and cooled
843 back to 20°C for fluorescence measurement. At 20°C EV1 is not permeable to the
844 dye, whereas temperature induced genome egress is detected as an increase in
845 fluorescence. White bar represent the behavior of SYBR Green II alone when
846 subjected to the described treatment.

847

848 **FIG. 3.** TEM images of negative stained EV1. (A) Intact EV1 virion. (B) EV1 heated
849 at 50°C for 3 minutes show protruding density from an otherwise structured particle.
850 (C) Heating for 10 minutes at 60°C result in complete particle disruption. Scale bars
851 are 25 nm and 100 nm for the left hand side and right hand side images,
852 respectively.

853

854 **FIG. 4.** Comparison of the Raman spectra of dried and aqueous EV1 particles. (A)

855 The full Raman spectra of dried intact EV1 virion. The most prominent vibrational
856 region is the aliphatic C-H stretching complex shown at around 2900 cm^{-1} which,
857 however, has a low information content of the structural details of the virion particles.
858 The fingerprint region locates between 700 and 1800 cm^{-1} . The sharp band at 2331
859 cm^{-1} originates from N_2 . (B) The fingerprint region of dried EV1 virion (top line) and
860 aqueous EV1 at 0.9 mg ml^{-1} and 3.8 mg ml^{-1} concentrations (middle and bottom
861 lines, respectively). Laser excitation with 532 nm and powers of 45 mW for dried and
862 200 mW for the aqueous viral samples were used with 30 exposures of 10 s in each
863 measurement. The spectra were normalized to the Phe vibration at 1003 cm^{-1} and
864 the baseline was shifted for better comparison of the spectra. All spectra were
865 measured at ambient temperature.

866

867 **FIG. 5.** The Raman spectra of aqueous EV1 particles at room temperature. On top,
868 the Raman spectra of intact EV1 (RT, black line), intermediate particles (50°C , blue
869 line) and disrupted particles (60°C , red line) are presented. Below, are the twofold
870 magnified difference spectra corresponding to the temperature intervals 60°C -RT
871 (red) and 50°C -RT (blue). The spectra are divided to individual sections and
872 normalized to mean signals of each section and the baselines were shifted for better
873 visualization. (A) $610\text{-}945\text{ cm}^{-1}$ (B) $945\text{-}1245\text{ cm}^{-1}$ (C) $1245\text{-}1520\text{ cm}^{-1}$, and (D) 1520-
874 1800 cm^{-1} . Marked vibrations from lower to higher frequency: 726 cm^{-1} , RNA; 783
875 cm^{-1} , RNA; 811 cm^{-1} , RNA phosphate symmetric stretching, an A form RNA helix
876 marker; 1003 cm^{-1} , Phe; 1096 cm^{-1} , a complex region of CC and CH vibrations of
877 lipid, RNA and protein capsid; 1127 cm^{-1} , non-aromatic amino acids and lipids; 1240
878 cm^{-1} , Am III; 1335 cm^{-1} , RNA bases; 1446 cm^{-1} , C-H vibrations from the proteins;
879 1478 cm^{-1} , RNA bases; 1572 cm^{-1} , Trp/RNA; 1668 cm^{-1} , Am I revealing information

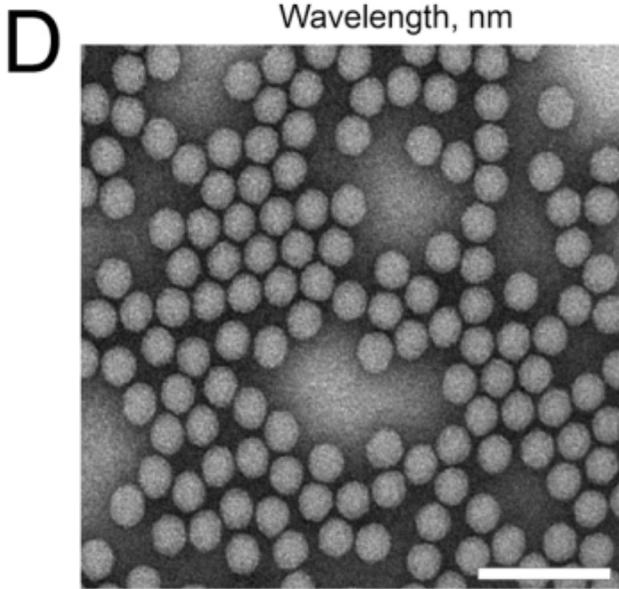
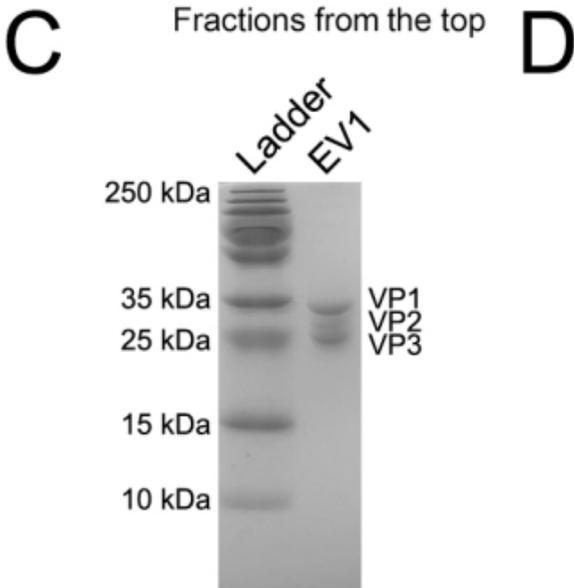
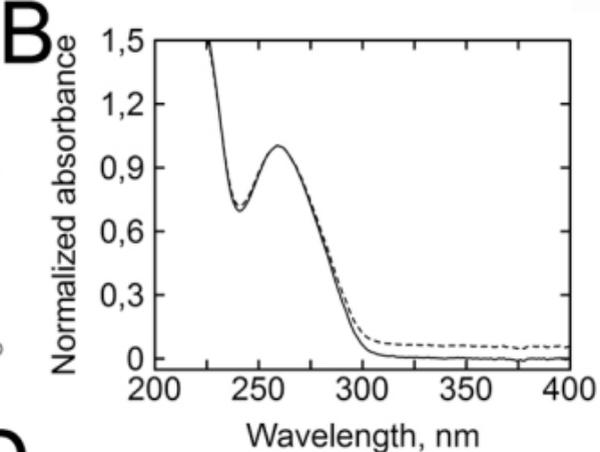
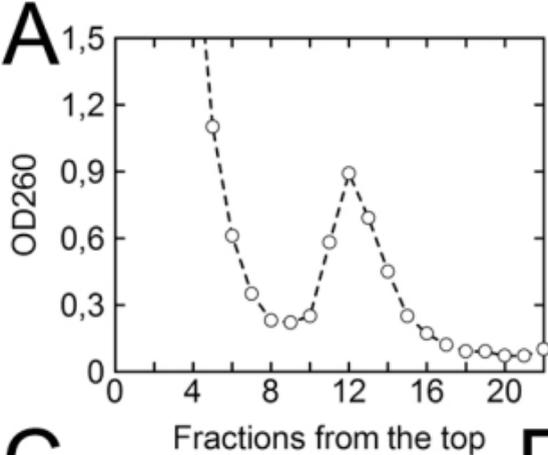
880 about the secondary structure of the proteins. The data acquisition was performed as
881 described in Fig. 4, and the spectra represent mean signals of measurements from
882 three different EV1 batches all with a concentration of about 3.8 mg ml⁻¹.

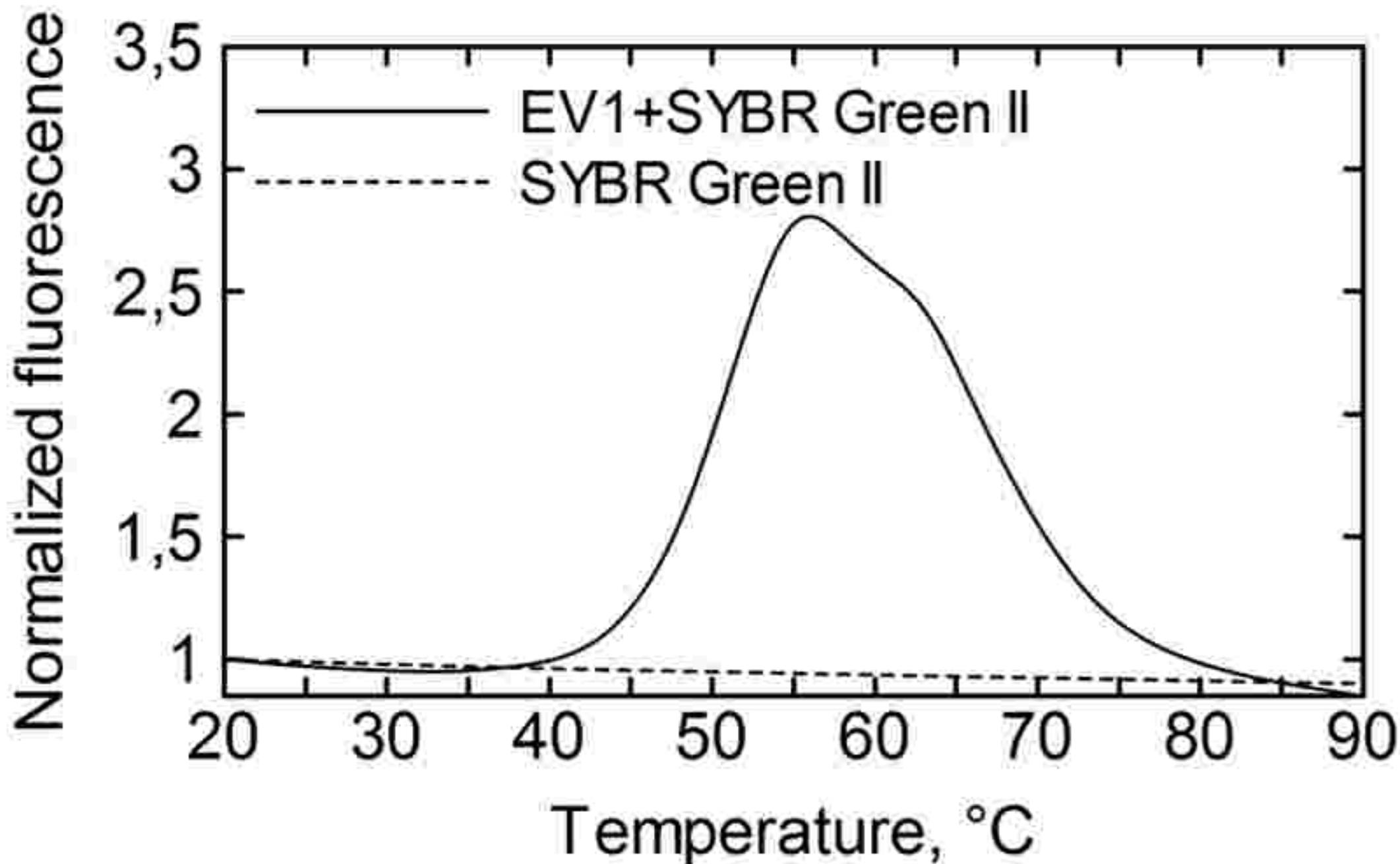
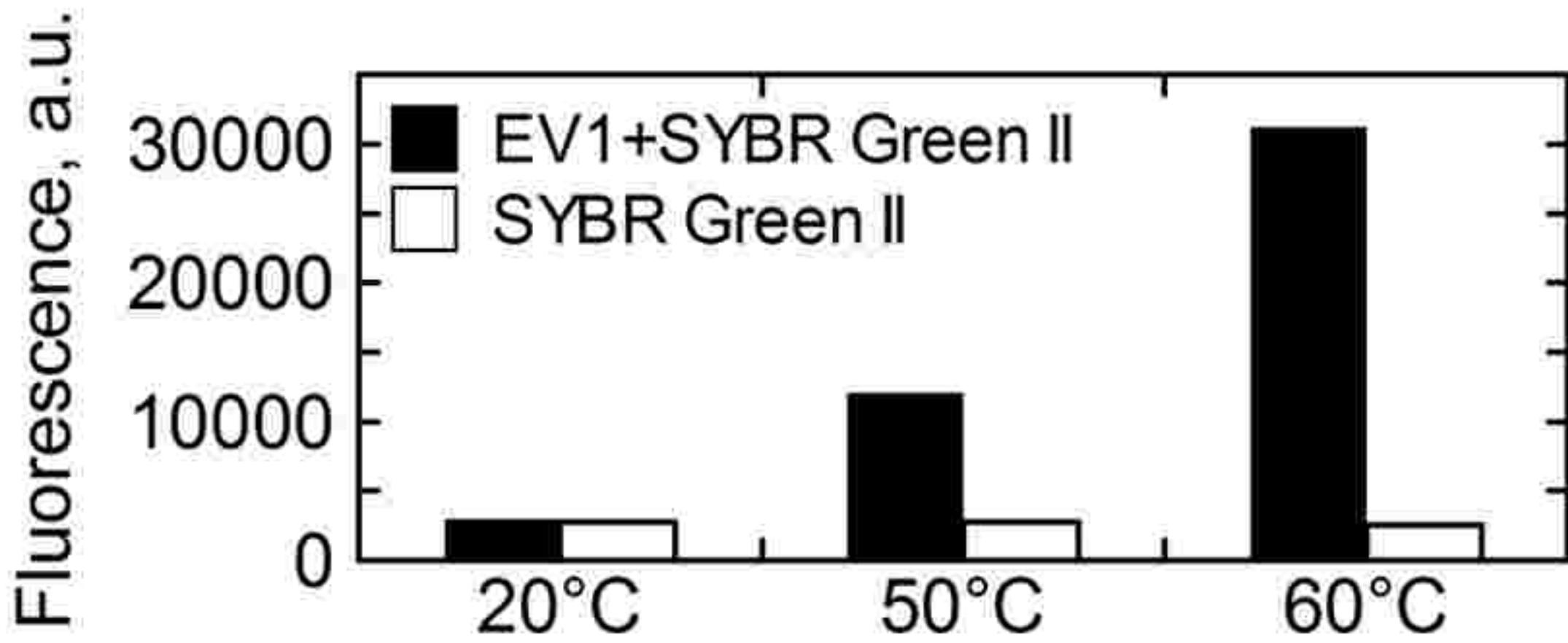
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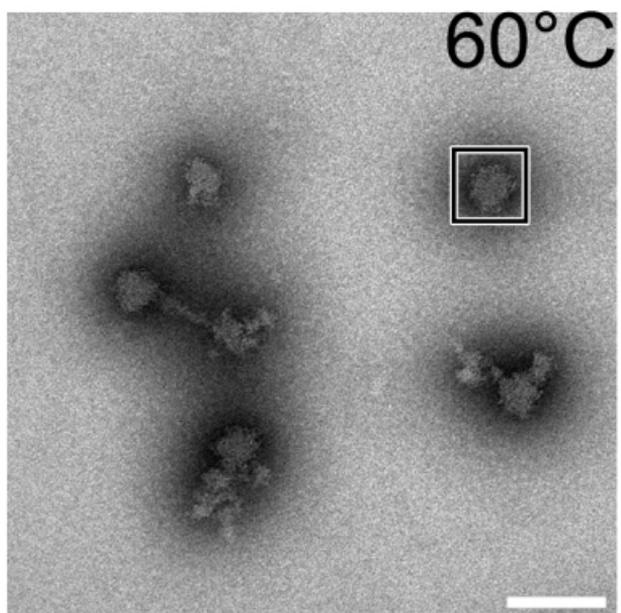
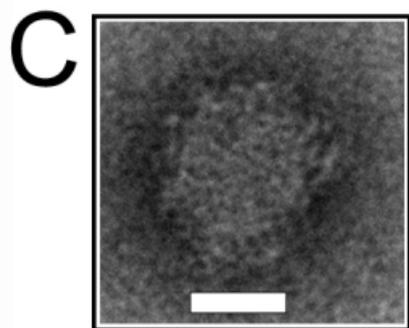
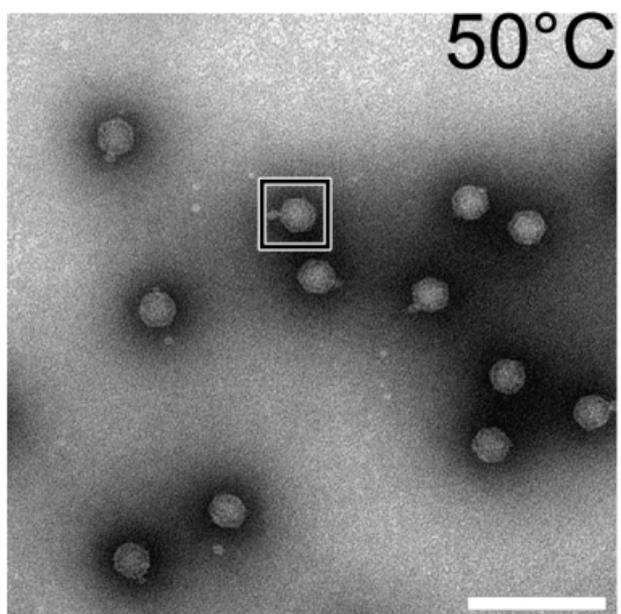
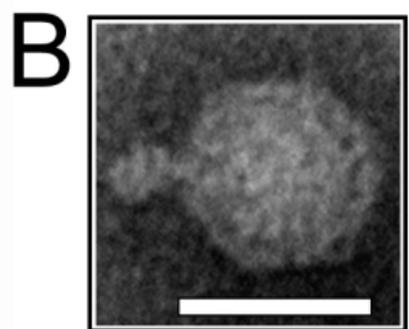
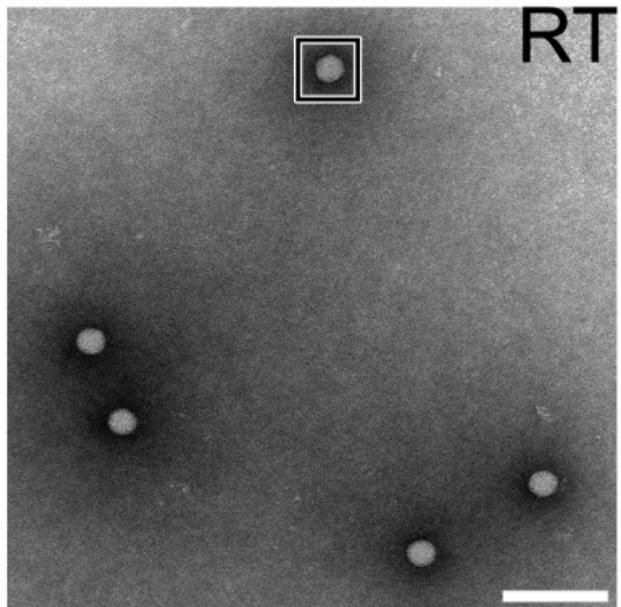
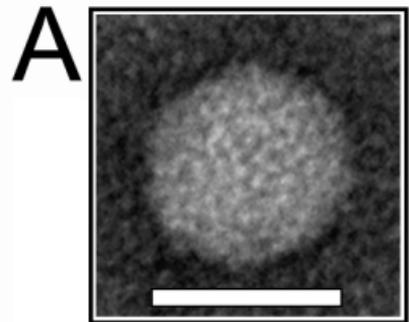
884 **FIG. 6.** Radial distribution of salt bridge donors in EV1 protomer. (A) Single protomer
885 (colored) positioning with respect to the whole capsid (half capsid displayed). (B) The
886 radial distribution of all atoms in EV1 protomer (PDB entry: 1EV1) are depicted in the
887 grey histogram to illustrate the density of the capsid shell. In color, VP1 (blue), VP2
888 (red), VP3 (green) and VP4 (yellow), and the positioning of the salt bridge forming
889 amino acids (Lys, Arg) in a single protomer, followed by their sequential positioning
890 within these structural proteins. (C) The distribution of the salt bridge donors shows
891 varying degree of connectivity within and between protomers. Interestingly, inner
892 protein VP4 is mainly forming connections between protomers and one inner VP-VP
893 connection.

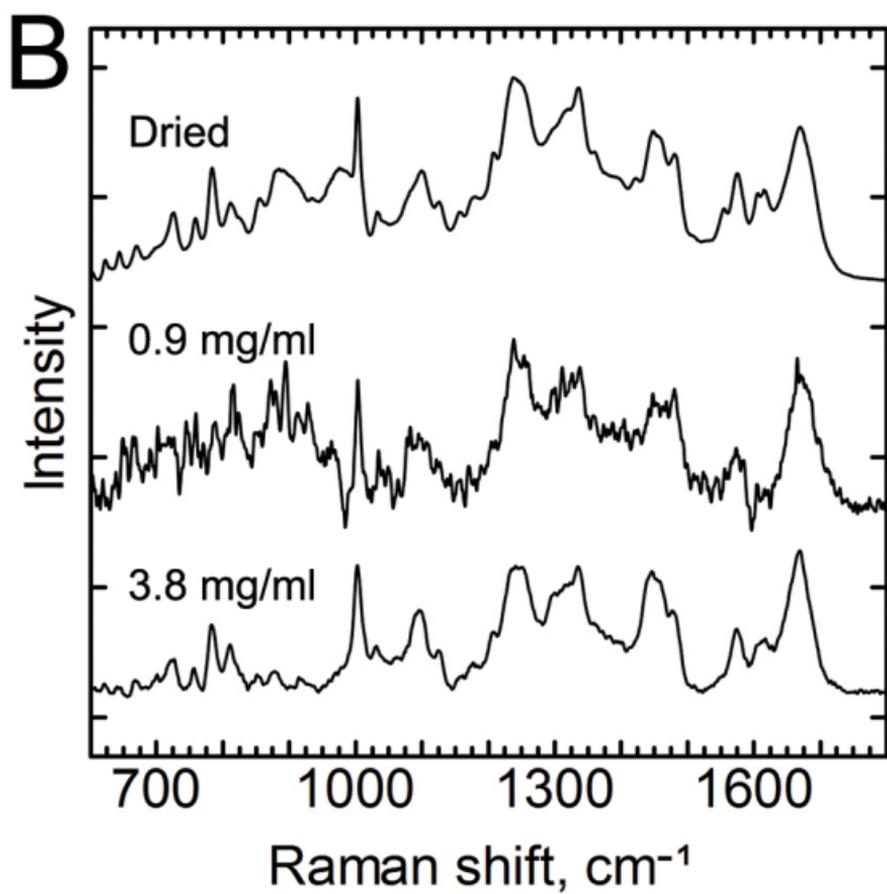
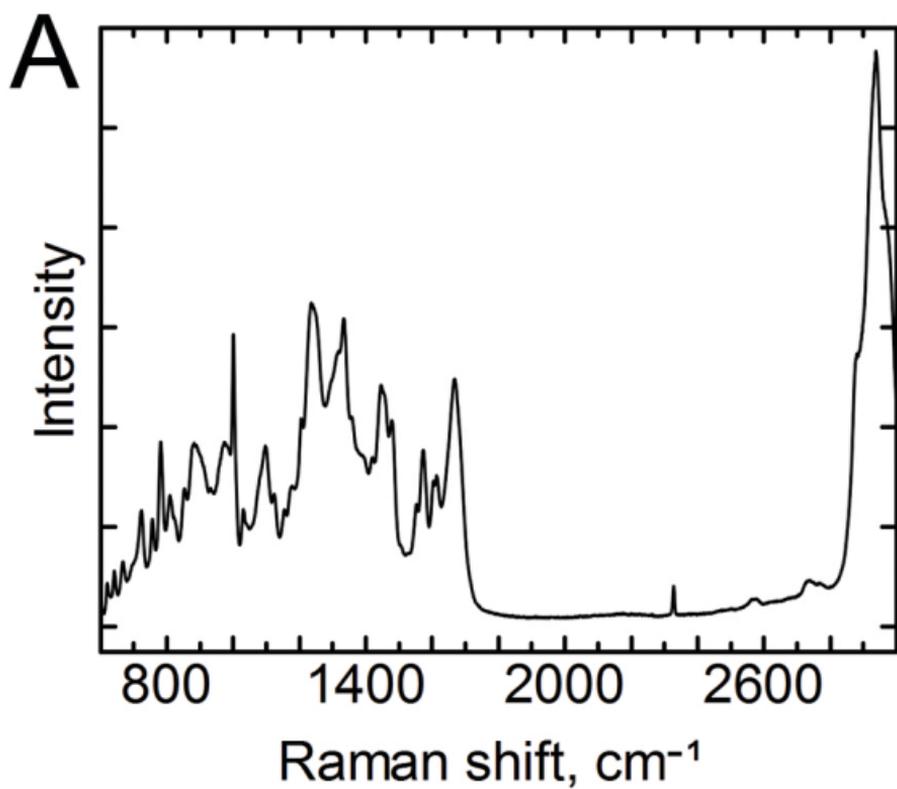
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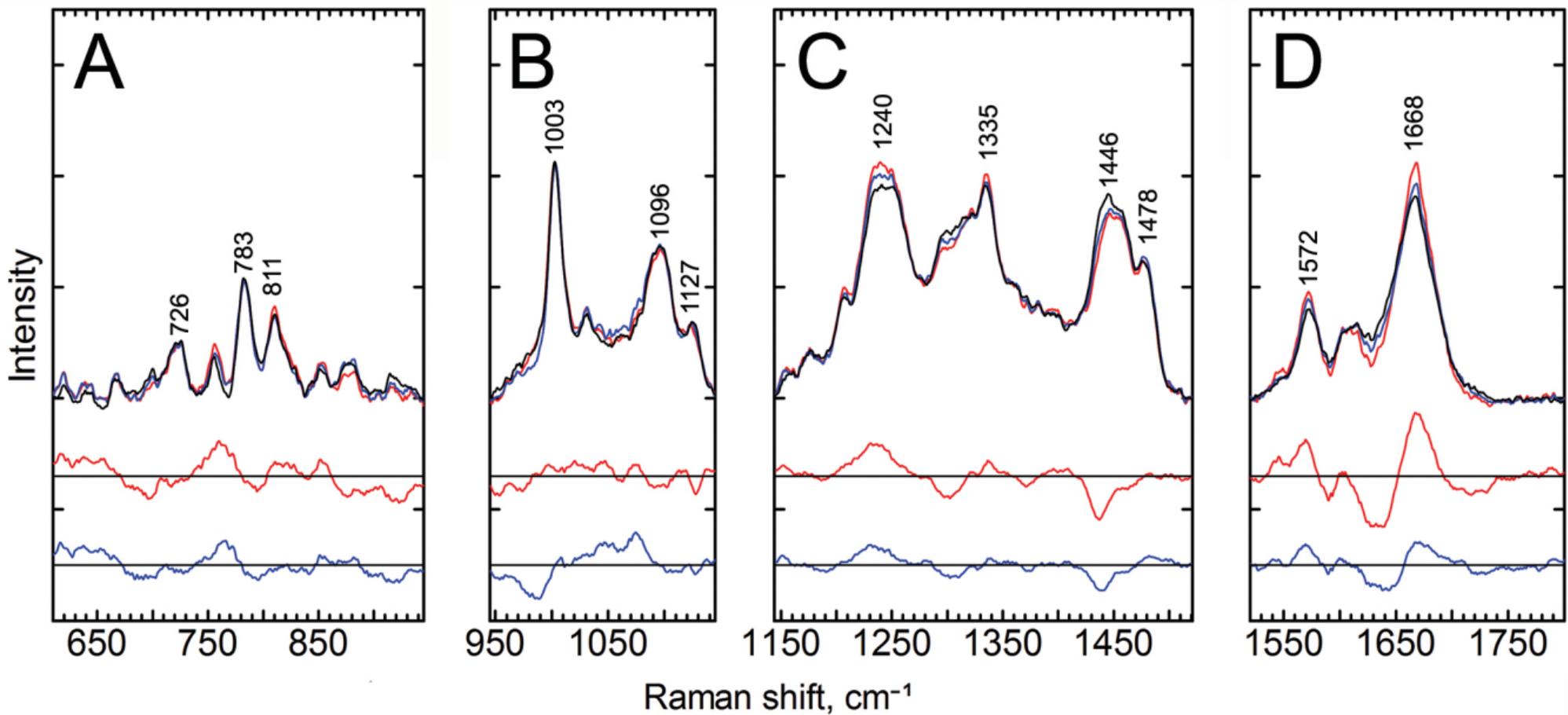
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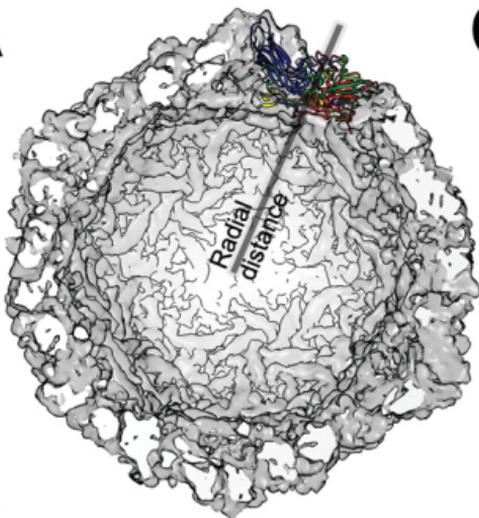
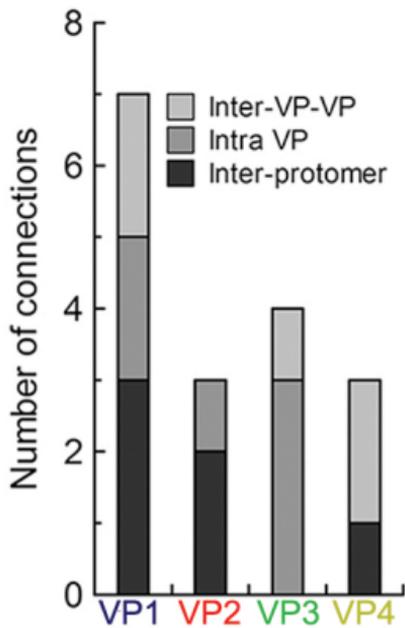


A**B**







A**C****B**