An unusual aspartic acid cluster in the reovirus attachment fiber $\sigma 1$ mediates stability at low pH

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1ABSTRACT

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4The reovirus attachment protein $\sigma 1$ mediates cell attachment and receptor binding and is
5thought to undergo conformational changes during viral disassembly. $\sigma 1$ is a trimeric
6filamentous protein with an α -helical coil-coiled Tail, a triple β -spiral Body, and a globular
7Head. The Head domain features an unusual and conserved aspartic acid cluster at the
8trimer interface, which forms the only significant intra-trimer interactions in the Head, and
9must be protonated to allow trimer formation.

10Here we show that all domains of $\sigma 1$ are remarkably thermostable across a wide range of
11pH, even at the low pH of the stomach. Interestingly, we determine the optimal pH for
12stability to be between pH 5-6, a value close to the pH of the endosome and of the jejunum.
13The $\sigma 1$ Head is stable at acidic and neutral pH, but detrimers at basic pH. When Asp³⁴⁵ in
14the aspartic acid cluster is mutated to asparagine, the $\sigma 1$ Head loses stability at low pH and
15is more prone to detrimers. Overall, the presence of the Body stabilizes the $\sigma 1$ Head.

16Our results confirm a role of the aspartic acid cluster as a pH-dependent molecular switch,
17and highlight its role in enhancing $\sigma 1$ stability at low pH.

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20INTRODUCTION

21

22Mammalian orthoreovirus (reovirus) infects humans during their childhood (1, 2), causing
23mild respiratory and enteric infection (3, 4). So far, three different reovirus serotypes with
24different tropisms and hemagglutination profiles have been discovered: T1L (Lang), T2J
25(Jones), and T3D (Dearing) (5-9). Reovirus enters epithelial cells via a two-step mechanism,
26first binding sialylated carbohydrates with low affinity, and then engaging receptor ssuch as
27Junctional Adhesion Molecule A (JAM-A)orNogo-66receptor (NogoR1)with high affinity (10-
2813) (14). After uptake, reovirus is internalized into the endosome where it is subjected to
29proteolytic cleavage of the outer capsid, leading to the production of infectious subvirion
30particles (ISVPs) (15, 16). In particular, cleavage of the outer capsid protein $\mu 1$ leads to
31exposure of hydrophobic sequences that mediate membrane penetration (17, 18).

32 Cellular uptake is mediated by the attachment protein $\sigma 1$: a trimeric, filamentous
33protein protruding from the vertices of reovirus (19, 20). $\sigma 1$ is anchored to the viral capsid at
34its N-terminus, followed by an α -helical coiled-coil region named Tail (21) and a tightly
35entwined Body domain featuring triple β -spiral repeats (22). The C-terminal Head region is a
36trimer of separately folded globular Head domains with a tight β -barrel fold, each composed
37of two Greek-key motifs (22). Both T1L and T3D $\sigma 1$ engage JAM-A with a conserved binding
38site at the base of their Head domains (11) (23). However, the carbohydrate receptor on host
39cells and its binding domains on $\sigma 1$ vary among different serotypes. The T3D Body domain
40binds to different terminal α -linked sialic acids (13, 22, 24, 25), while T1L $\sigma 1$ engages the
41sialylated branched GM2 glycan with its Head domain (26).

42 Upon transition from virions to ISVPs, the attachment protein $\sigma 1$ undergoes
43conformational changes (20) (27, 28) from a contracted form to a more elongated
44conformation, maybe the one observed in the crystal structures (22, 26, 29).However, neither
45the nature nor the trigger for these conformational changes is entirely understood.
46Candidates are carbohydrate binding (30), pH, and proteolytic cleavage (25). The binding of

479BG5 monoclonal antibody to virions but not to ISVPs, has been suggested as the evidence
48for a conformational change on the virion (20) (27). In addition, the binding of sialic acid
49leads to higher affinity of the virus for JAM-A (30).

50 An attractive candidate for a molecular switch is the highly conserved cluster of
51aspartic acid residues at the trimer interface at the bottom of the globular Head (31, 32). In
52T3D $\sigma 1$, three copies of aspartic acids Asp³⁴⁵ and Asp³⁴⁶each are buried in a hydrophobic
53and solvent-inaccessible pocket (10, 33).The side chains of protonated Asp³⁴⁵ form hydrogen
54bonds with the deprotonated side chains of Asp³⁴⁶ on the clockwise neighbouring monomer
55within the trimer. Evidence for the unusual protonation of Asp³⁴⁵ at neutral pH comes from a
56variant, in which Asp³⁴⁵ was replaced with neutral asparagine that cannot be deprotonated.
57The D345N $\sigma 1$ Head formed trimers at neutral pH, bound JAM-A with similar affinity to the
58wild-type, and exhibited a highly similar overall structure (10).The aspartic acid cluster is
59shielded from the solvent by two layers of aromatic amino acids directly below and above the
60aspartic acid residues, likely protecting the protonated aspartic acid cluster at neutral pH.
61Both the aspartic acids and the neighbouring residues are highly conserved among different
62prototype and field isolate reovirus strains, indicating an important role for reovirus infection.
63Formation of the trimer of $\sigma 1$ Head domains from monomers likely needs to take place in a
64relatively low pH environment that enables at least partial protonation of Asp³⁴⁵ in the
65monomer. Therefore, it is likely that these residues could play a role in inducing $\sigma 1$
66conformational changes, during the infection and the cell entry process (10, 33).

67 Reovirus encounters low pH in the endosome during infection of host cells, but it is
68also exposed to drastically varying pH during transmission and initial infection of a host
69organism. Following ingestion of contaminated food or inhalation of aerosols, reovirus travels
70to the gastrointestinal tract, encountering the stomach and the small and large intestines (34,
7135). These environments provide different pH ranges, oxygen levels, and bacterial loads (36-
7238). While the pH is constantly acidic (1.5 – 3.5) in the stomach, the transition to the small
73and large intestine leads to shift towards mildly acidic and neutral pH ranges (6.3 – 7.3) (36,

7439). Reovirus needs to withstand the low gastric pH and might use low pH as a trigger for
75conformational changes. However, there is no direct evidence on how changes in
76environmental pH can affect $\sigma 1$ conformation, stability, and trimeric organization. In this
77study, we conducted a biophysical investigation on the thermostability and the
78oligomerization state of recombinant $\sigma 1$ domains over a broad range of pH, including the
79very low pH of the stomach. We expressed and purified constructs spanning all of T3D $\sigma 1$.
80We then determined their melting temperatures via differential scanning fluorimetry,
81identifying the pH of optimum stability of T3D $\sigma 1$ constructs to be pH 5, a value close to the
82pH of the endosome and of the jejunum. We found by analytical size exclusion
83chromatography that the $\sigma 1$ Head detrimers at high pH, confirming protonation of the
84aspartic acid cluster. The effect of the D345N mutation at the trimer interface was found to
85particularly affect the stability of $\sigma 1$ trimer at neutral and low pH, upon incubation at room
86temperature. Altogether, our findings suggest that the aspartic acid at the trimer interface of
87the $\sigma 1$ Head domain is required for the stability of the trimeric Head domain, especially in a
88low pH environment.

89

90EXPERIMENTAL PROCEDURES

91

92**Protein expression and purification.** T3D σ 1 Tail-Body (25-291) construct was a kind gift
93of the Stehle laboratory (UniversitätTübingen). T3D σ 1 Tail-Body was subcloned into
94pETM11 vector in frame with a N-terminal tag consisting of a hexahistidine (His) motif for
95affinity purification and a Tobacco Etch Virus (TEV) protease cleavage site for removal of the
96His-tag during purification (40). After cleavage of Tail-Body, the amino acids GAMA remained
97at the N-terminus.

98 Body-Head (170-455) and Head (293-455) constructs, which lack the stably trimeric
99Tail, had been subcloned into a modified pQE80L vector and additionally provided with a
100trimeric version of the GCN4 leucine zipper to induce trimerization (41, 42). After cleavage,
101the amino acids GAMA remained at the N-terminus of the cleaved protein. Site-directed
102mutagenesis was used to produce T3D D345N σ 1 in the context of the Body-Head and Head
103constructs.

104 Upon transformation into BL21(DE3)-RIL cells, protein was expressed by
105autoinduction (43) at 20 °C for 4 days. Bacteria were harvested by centrifugation and the
106bacterial pellet was resuspended in 40 mM HEPES pH 7.1, 300 mM NaCl, and 10 mM
107Imidazole, supplied with 1 mM PMSF, 0.01 mg/mL DNase I, and 0.2 mg/mL Lysozyme. Cells
108were lysed with an Emulsiflex-C5 Homogenizer (Avestin) and the insoluble fraction was
109removed by centrifugation at 21,500 X g. The soluble fraction was purified via immobilized
110metal affinity chromatography (IMAC) on a HisTrapTMFF column (GE Healthcare) and eluted
111with a linear gradient to 40 mM HEPES pH 7.1, 300 mM NaCl, 500 mM Imidazole. Controlled
112TEV-protease treatment was performed during dialysis against 40 mM HEPES pH 7.1, 300
113mM NaCl, 10 mM Imidazole at room temperature for 4 hours, in order to cleave the 6X-
114HisTag and simultaneously reduce the Imidazole concentration. The cleaved protein was
115isolated via reverse mode IMAC and further purified by gel filtration on a HiLoad[®] 16/600
116Superdex[®] 200 pg in 40 mM HEPES pH 7.1, 300 mM NaCl. The pure protein was either used

117freshly or cryoprotected by the addition of 20% (v/v) ethylene glycol, flash-frozen in liquid
118nitrogen and stored at -80°C .

119

120**Differential scanning fluorimetry.** Differential scanning fluorimetry was performed adapting
121the protocol from Niesen F.H. et al. (44). T3D $\sigma 1$ constructs were thawed and dialyzed
122multiple times on ice with a ZelluTrans/Roth Mini Dialyzer (Roth) against 40 mM HEPES pH
1237.1, 150 mM NaCl, to remove the cryo-protectant. They were then diluted to 0.2 mg/mL and
124mixed with SYPRO orange (Sigma-Aldrich) to give a final dye:protein molar ratio of 1:500.
125T3D $\sigma 1$ constructs were tested against buffers in a range of pH between 1.4 and 9.4 (Table
126S1). In a 96-well plate, 10 μL of the different assay buffers (0.4 M) were mixed with 10 μL of
127 $\sigma 1$ protein to give final buffer and protein concentrations of 0.2 M and 0.1 mg/mL,
128respectively. Melting curves were recorded in triplicate on a Mx 300P qPCR system (Agilent),
129between 25°C and 95°C , with a scanning rate of $1^{\circ}\text{C}/\text{min}$. Data was fitted to the Boltzmann
130equation with Origin[®] Pro 9.5.

131

132**Analytical gel filtration.** T3D $\sigma 1$ constructs were dialyzed at room temperature with
133ZelluTrans/Roth Mini Dialyzers (Roth) against buffers titrated to pH 3.0, 5.0, 7.1 and 8.6
134(Table S2), to eliminate the cryo-protectant and equilibrate the sample to the assay pH. T3D
135 $\sigma 1$ samples were tested before and after an additional 4-hour incubation step at room
136temperature. 50 μL of 0.4 mg/mL $\sigma 1$ protein were run on a Superdex[®] 200 Increase 3.2/300,
137at a flow rate of 0.075 mL/min while monitoring the protein absorbance at 280 nm. Elution
138peaks corresponding to trimers and monomers were integrated in Unicorn (GE Healthcare,
139Cytiva).

140RESULTS

141

142T3D $\sigma 1$ is stable over a wide range of pH values.

143 To determine the effect of pH on the stability of different $\sigma 1$ domains, we
 144recombinantly expressed and purified three different constructs spanning the entire length of
 145 $\sigma 1$ (Fig. 1A): a Tail-Body construct spanning the coiled-coil Tail as well as most of the Body
 146domain (25-291), a Body-Head construct containing the entire body domain as well as the
 147Head domain (170-455) and the Head domain with the last β -spiral (293-455). All $\sigma 1$ wild-
 148type constructs were purified as trimers at pH 7.1. To assess the effect of pH on protein
 149stability, we incubated the $\sigma 1$ constructs with an excess of buffers of different pH ranging
 150from 1.4 to 9.4 in increments of 0.5 pH units (Table S1) and acquired thermal denaturation
 151curves at each pH by differential scanning fluorimetry. While most proteins evolved against
 152thermal denaturation under physiological conditions, the melting temperature reports on the
 153overall stability of a protein domain – the higher the melting temperature, the more rigid and
 154stable the protein domain. Thus, melting temperatures also report on changes in protein
 155conformations or dynamics under different conditions. As reovirus travels through the
 156stomach and gastrointestinal tract, we hypothesized that $\sigma 1$ constructs might be more stable
 157in a low pH environment.

158 All T3D $\sigma 1$ constructs featured high thermostability at low pH values between 1.4 and
 1595, with melting temperatures between 45 and 70 °C (Fig. 1C-E). In particular, all constructs
 160have a melting temperature much higher than 37 °C even at the highly acidic pH of pH 1.4,
 161with 53, 45 and 50 °C for the Tail-Body, Body-Head, and Head constructs, respectively. For
 162comparison, the acid-unstable model protein Glutathion-S-transferase (GST) instantly
 163denatured at temperatures lower than 25 °C at pH values of 4 and below (Fig. 1B). The
 164unfolding signal of the Tail-Body construct is strongly determined by unfolding of the Body
 165domain as the Tail domain does not contain aromatic amino acids to which the dye used for
 166detection could bind.

167 When comparing melting temperatures across the pH spectrum, the Tail-Body
168construct featured a single transition, with constant melting temperatures of roughly 50 °C
169between pH 1.4 and 4, and constant melting temperatures of roughly 60 °C between pH 5
170and 9.4 (Fig. 1C). In contrast, the Head domain featured a stability optimum of 58 °C
171between pH 5 and 6, with a gentle drop in stability at lower pH and a steep drop in stability at
172higher pH, leading to a melting temperature of only 38 °C at pH 9.4 (Fig. 1E). A melting
173temperature of 38 °C was the lowest value acquired among all the constructs tested and the
174closest to the physiological temperature of the human body. The Body-Head construct was
175more stable than the Tail-Body or the Head constructs, with a broad pH optimum of stability
176of 67-70 °C between pH 5 and 8.4, while Tail-Body and Head constructs featured 10 °C
177lower melting temperatures at pH 5. In addition, the Body-Head construct featured deep
178drops in stability towards both low and high pH: a moderate drop towards lower pH values
179and a steep drop to about 48 degrees between pH 8.5 and 9 (Fig. 1D). When tested for its
180thermostability in a various range of pH, the T3D σ 1 Body-Head construct showed overall
181higher melting temperatures than the Head domain expressed alone. Although the melting
182temperature values were comparable between the two constructs in the low pH of the
183stomach, at the optimum pH of 5, their difference was about 10 °C. The same gap in melting
184temperatures could also be detected upon incubation with basic pH environment. In addition,
185from pH 5 onwards, the Head construct showed a sharp decreasing in melting temperatures,
186reaching its lowest value of 37 °C, at pH 9.4. Interestingly, when the Body domain was
187expressed together with the Head domain, its overall stability was enhanced and the lowest
188melting temperature detected was 47 °C, at pH 9.4.

189 Taken together, the stability optimum of all constructs lays around pH 5-6, which
190interestingly coincides with the pH of the endosome as well of the upper gut. In addition,
191while all T3D σ 1 constructs were stable at acidic pH, there were differences between the
192constructs upon incubation in neutral and basic buffers. Consequently, we conclude that the
193different domains of the elongated σ 1 molecule are differentially affected by changes in
194environmental pH. While the increase in pH did not influence the melting temperature of the

Body-Tail construct, it caused significant destabilization of the Head and Body-Head constructs. We therefore hypothesized that the high-pH drop in thermostability of the constructs containing the Head domain was caused by the Head domain, which features an aspartic acid cluster at the trimer interface that had been proposed to act as a pH-sensing switch(10).

200

201The Head domain of T3D σ 1 detrimerizes at high pH.

202 The highly conserved aspartic acid cluster and its surrounding structures at the bottom of the σ 1 Head domain are the main trimerization interface of the σ 1Head(10). Based on the high-resolution crystal structure of the σ 1 Head, it was hypothesized that the trimer of σ 1 Head domain can only be formed and maintained if Asp³⁴⁵ in the cluster interface is protonated. At neutral pH, protonation of Asp³⁴⁵ can only be maintained by shielding it from solvent. However, if thermal motion or a basic environmental pH overcome the shield and Asp³⁴⁵ is then deprotonated, repulsion between the negatively charged Asp³⁴⁵ residues would cause the σ 1 Head to detrimerize.

210 Based on our thermostability data, which showed changes in stability with pH, we therefore investigated the multimeric state of the σ 1 Head domain at different pH values by analytical size exclusion chromatography, which reports on the hydrodynamic radius of macromolecules. We used homogeneous purified trimeric σ 1 Head and incubated it for 4 hours at RT in the respective pH before analysis. After analytical size exclusion chromatography, the amount of monomeric T3D σ 1 was calculated as a fraction of the total loaded protein. At acidic and neutral pH values, the wild-type Head construct was almost exclusively trimeric, even after incubation for 4 hrs at RT. At pH 8.6, the wild-type Head partially dissociated into monomers, leading to a 25% fraction of monomeric wild-type Head. Our results without incubation are in accord with previous findings indicating the wild-type σ 1 Head to be trimeric when analysed at 4°C and without incubation (10). However, our experiments were performed at 20 °C or higher, which more closely resembles physiological

conditions and provides increased thermal motion leading to more dynamic protein structures (45).

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226

D345N T3D σ 1 mutant Head is overall less thermostable than the wild-type Head.

228 To investigate the role of the protonated aspartic acid cluster in T3D σ 1 Head
229stability, we replaced Asp³⁴⁵ with an asparagine to mimic a permanently protonated aspartic
230acid.

231While aspartic acid features a carboxylic acid group (-COOH), asparagine carries an amide
232group (-CONH₂) in which one of the oxygens is replaced with a nitrogen atom. Due to this
233chemical difference, free aspartic acid is deprotonated and negatively charged at neutral pH,
234while asparagine is not deprotonated even at basic pH. Moreover, asparagine can engage in
235the same pattern of hydrogen bonds as protonated aspartic acid. As asparagine cannot be
236deprotonated, we anticipated the D345N σ 1Head to be more resistant to thermal
237denaturation and essentially more stable at the trimer interface than the wild-type at neutral to
238basic pH.

239 The D345N σ 1 Head construct featured similar melting temperatures to that of the
240wild-type, with roughly 43°C at mildly acidic, neutral and basic pH. However, the D345N σ 1
241Head construct showed no drop-off in stability at basic pH, in contrast to the wild-type (Fig.
2422A). The finding confirmed our hypothesis on the role of D345. However, unexpectedly, the
243overall melting temperatures of the D345N mutant Head were much lower than those of the
244wild-type construct across pH 1.4 to pH 8.4. Compared to the fairly stable wild-type Head,
245D345N Head had a 10-15 °C lower melting temperature at mildly acidic and neutral pH, and
246a 25 °C lower melting temperature at very low pH. In particular at pH 1.4-3.4, the melting
247temperatures of the D345N mutant were 10°C below physiological temperature, indicating
248that it might be unstable at gastric pH. Meanwhile, the wild-type Head construct was fairly
249thermostable at very low pH.

250

251 **D345N T3D σ 1 Head detrimers into monomers.**

252 We then asked whether the decreased thermostability of the D345N Head correlated
253 with its multimeric state and performed analytical gel filtration at different pH values. At mildly
254 acidic and neutral pH, a 10 % fraction of the D345N mutant Head dissociated into monomers
255 (Fig. 2A). Interestingly, at pH 3.0, D345N had completely dissociated into monomers during
256 the buffer exchange and no further detrimers was observed during incubation at room
257 temperature. In contrast, the wild-type constructs had been almost exclusively trimeric at
258 acidic and neutral pH (Fig. 2A). The decreased stability of the D345N Head trimer at acidic
259 pH correlates with the decreased thermostability of the protein in the same pH range.
260 Moreover, the wild-type protein was predominantly trimeric at acidic pH and much more
261 thermostable. Therefore, the D345N mutation likely caused the decreased thermostability by
262 disrupting the trimerization interface at acidic pH. Our results therefore point to a role for the
263 highly conserved aspartic acid in the trimerization interface in stabilizing the trimer at low pH.
264

265 **Structural basis for the lower stability of D345N mutant Head.**

266 The D345N mutation did not change the charge, the packing of side chains, nor the
267 pattern of hydrogen bonds as D345 is protonated, but it did alter charges in the trimer
268 interface. Yet, the variation had a strong effect on trimer integrity and overall protein stability.
269 To elucidate its mechanism, we compared the previously determined high-resolution crystal
270 structures of wild-type and D345N σ 1Head (PDB accession codes: 2OJ5 and 2OJ6,
271 respectively)(10).

272 The wild-type and D345N proteins had crystallized in the same space group with
273 identical unit cell parameters, the same two trimers in the asymmetric unit and diffracted to
274 similar resolution of 1.75 and 1.85 Å resolution, respectively. The overall structures were
275 virtually identical with main chain r.m.s.d. value of 0.13 Å, consistent with the fact that the two
276 proteins differ in only one heteroatom. However, there was a significant difference between
277 the two variants in the distance between the side chain of residue 345 and its hydrogen

278 bonding partner across the trimer interface, Asp^{346cw} (cw indicating the clockwise neighboring
279 monomer in the $\sigma 1$ trimer). This distance averaged 2.54 Å among the six crystallographically
280 independent monomers in the asymmetric unit in the wild-type crystal, but it averaged 2.93 Å
281 in the D345N crystal (Fig. 2C). Thus, this hydrogen bond was 0.4 Å longer in the D345N
282 mutant than in the wild-type protein (statistically significant with $p=1.7 \times 10^{-5}$ by Welch t-test).
283 The length of hydrogen bonds inversely correlates with their strength (46-50), with 2.5 Å
284 indicating a very strong bond and 3.5 Å often used as a generous cutoff for weak hydrogen
285 bonds. Thus, the wild-type trimer is held together by a much stronger hydrogen bond than
286 the D345N mutant.

287

288 **The addition of the Body to the Head domain enhances stability.**

289 On the virus, the globular $\sigma 1$ Head domain is linked to the $\sigma 1$ Body domain, which
290 overall stabilized the Head domain (Fig. 1D,E) and might counteract the D345N mutation.
291 We therefore tested the effect of the D345N mutation on the stability of the Body-Head
292 construct. Similar to the D345N Head alone, the overall thermostability of D345N Body-Head
293 did not decrease at high pH (Fig. 3A), while the thermostability of the wild-type Body-Head
294 construct was reduced in a basic environment. However, the overall melting temperatures of
295 the D345N mutant Body-Head were 10 °C lower than those of the wild-type construct at
296 acidic and neutral pH, confirming the stabilizing effect of Asp³⁴⁵ in the wild-type protein.
297 Again, the D345N mutant Body-Head construct was least stable at very low pH, reaching its
298 lowest value of 32 °C at pH 1.4. When comparing D345N Head and Body-Head domains, the
299 addition of the Body to the Head domain stabilized the protein overall to give 15 °C higher
300 melting temperatures across the pH range tested.

301 We then asked whether the addition of the Body domain prevented detrimerization of
302 the wild-type and D345N Head at low pH and determined its oligomeric state by analytical
303 size exclusion chromatography. Interestingly, the wild-type Body-Head construct was present
304 almost exclusively as a trimer after incubation in different pH buffers, both directly after buffer
305 exchange and after 4 hrs incubation at room temperature (Fig. 3B). The D345N Body-Head

306construct showed some detrimerization at pH 3.0 (15% monomer) and after 4 hrs incubation
307at pH 8.6 (8% monomer). Therefore, the Body domain prevented detrimerization of the wild-
308type protein at high pH (Fig. 2B, 3B) and greatly reduced detrimerization of D345N at low pH.

309

DISCUSSION

311

312 $\sigma 1$ is thought to adopt a contracted conformation on the virion and is hypothesized to
 313 undergo a conformational change to a more elongated form during virus entry (20, 27). An
 314 antibody against the outer capsid protein $\sigma 3$ prevents hemagglutination of red blood cells,
 315 which is mediated by the sialic acid binding site located in the body domain of $\sigma 1$. Thus,
 316 either the Body or the Head domain of $\sigma 1$ is likely close to the virus surface in the contracted
 317 form. The recombinant $\sigma 1$ domains used in this study are all elongated (22, 29) and therefore
 318 are thought to represent the structure of $\sigma 1$ after the conformational change. While the Body
 319 domain seems to feature a relatively flat pH profile of thermostability, the $\sigma 1$ Head is the only
 320 domain whose thermostability is strongly influenced by pH. Even at strongly acidic pH, wild-
 321 type $\sigma 1$ Head is a highly stable trimer, with no detrimerization occurring and a pH optimum of
 322 thermostability of the protein between pH 5 and pH 6. This is likely an adaptation of the
 323 protein structure to the exposure to low intragastric pH during transmission. Similarly,
 324 structural studies of the enteric adenovirus Ad-F41 short fiber head revealed a loop in the
 325 fiber head that was disordered at neutral pH and ordered at pH 5 (51, 52). Moreover,
 326 comparison of the Ad-F41 capsid to that of non-enteric adenoviruses highlighted a surface
 327 with relatively few charged residues and an almost unaltered structure at pH 4 (53)

328

329 We find here that the thermal motion at room temperature combined with a basic pH
 330 of 8.6 causes slow detrimerization of the wild-type $\sigma 1$ Head. This result directly confirms in
 331 solution earlier conclusions from the crystal (10, 33) that the $\sigma 1$ Head trimer might be labile
 332 to high pH. Earlier molecular dynamics simulations suggested that deprotonation of Asp³⁴⁵
 333 causes opening of the Head trimer by electrostatic repulsion (33). Moreover, removing the
 334 hydrophobic shielding from the aspartic acid cluster of $\sigma 1$ led to purification of a monomeric
 335 $\sigma 1$ Head construct, likely by allowing direct deprotonation of Asp³⁴⁵ (10). Thus, a likely
 336 mechanism for detrimerization could be that the $\sigma 1$ Head undergoes opening and closing
 337 “breathing” motions due to thermal motion, which might lead to deprotonation of Asp³⁴⁵ at

basic pH. This model is consistent with the crystal structure featuring solid hydrophobic interactions between monomers at the bottom of the Head and weaker interactions at the top of the Head (10). However, at acidic and neutral pH, complete detrimerization of the wild-type $\sigma 1$ Head was negligible, indicating that the trimer was stable under those conditions. This result, however, does not rule out that the $\sigma 1$ Head might undergo opening and closing breathing motions at neutral without deprotonation of Asp³⁴⁵ and complete detrimerization. Our gel filtration assay cannot distinguish between open and closed trimer conformations of a small domain such as the $\sigma 1$ Head.

To investigate the role of Asp³⁴⁵ as a pH-dependent switch, we then replaced Asp³⁴⁵ at the centre of the trimer interface with asparagine, which cannot be deprotonated and engages in the same pattern of hydrogen bonds as protonated aspartic acid. The biggest effects of the mutation were observed at acidic pH: a considerable decrease in overall thermostability correlating with an increased tendency to detrimerize. Even at its pH optimum of stability, the D345N mutant had a 15 °C lower melting temperature than the wild-type. This effect was even more pronounced at pH 3, which the virus might encounter in the stomach.

As the stability of the D345N trimer depended on the pH of incubation, it is likely that there are ionizable residues in the $\sigma 1$ Head that destabilize the trimer at low pH. One candidate is His³⁴⁹, three copies of which are located on the inner surface of the trimer just above the top of the aspartic acid sandwich and whose pK value lies in the relevant pH range. It is thus likely that the strong interactions in the aspartic acid sandwich hold the trimer together against pH-dependent repulsion in other parts of the protein. This hypothesis argues for an equilibrium of forces at the trimer interface of $\sigma 1$.

Inspection of the previously solved wild-type and D345N crystal structures provide a framework to understand the observed differences in trimer stability (10). The -COOH to -OOC-hydrogen bond mediated by the protonated Asp³⁴⁵ to the deprotonated side chain of Asp³⁴⁶ on the clockwise neighbouring monomer within the trimer is quite short, with a distance between the two oxygen atoms involved of 2.5 Å. Short hydrogen bonds correlate

with increased bonding strength (54). In the D345N mutant, the corresponding -CONH₂ to -COO⁻-hydrogen bond is 0.4 Å longer, which correlates with a much weaker hydrogen bond (54). Thus, it is likely that a protonated D345 can hold a marginally stable trimer together, even at low pH and elevated temperatures, while the bonding strength of N345 might not be enough. Interestingly, the corresponding bond in the 2.2 Å crystal structure of the T1L σ1 Head (55) is even shorter with an average length of 2.4 Å. Thus, the increased bonding strength of the aspartic acid cluster is likely a conserved feature among different reovirus serotypes.

When the Head domain was expressed together with the Body portion, we observed enhanced thermostability across the pH range. Likewise, the Body-Head domain seemed to almost fully prevent detrimerization of the Head domain. We envisage a model in which a relatively pH-invariant Body domain prevents detrimerization of the Head, which is more sensitive to changes in pH. While the Head purified alone can detrimerize readily according to pH, Body-Head trimers might undergo motions of the Head domain, but do not detrimerize completely when linked to the Body domain.

We find that lower thermostability of σ1 head correlated with a higher propensity to detrimerize into monomers. This result suggests that monomeric σ1 Head is less thermostable than the trimer. Interestingly, a σ1 Head mutant that was purified as a monomer was unable to recognize JAM-A (10) even though JAM-A contacts only one σ1 monomer and the mutation was not in the σ1-JAM-A interface. These results can be explained with decreased stability of the monomeric mutant causing a loss of function. Likewise, in the context of the virus, T1L Reovirus bearing disulfide crosslinks between the monomers in the Head domain had higher avidity for its receptor JAM-A than virus without crosslinks (56). Furthermore, enhanced stability of the σ1 trimer at the Head domain correlated with increased plaque size. Presumably, σ1 on virus without crosslinks can partially detrimerize at the Head, with a loss of stability and reduction in JAM-A binding. Moreover, not all crosslinked viruses featured all three possible crosslinks in the Head

domain, indicating that the Head domain was not as rigidly trimeric as the body or tail domains.

Trimer-monomer dynamics have lately been observed for several trimeric viral surface proteins, most prominently pre-fusion conformations of viral class I fusion proteins. For example, antibodies which target the inner surfaces of the trimer interface of influenza virus hemagglutinin are produced in infected and vaccinated individuals and confer protection in vivo. Binding assays suggest that the inter-trimer interface is at least temporarily accessible to these antibodies and can be disrupted by them, most prominently in the uncleaved HA0 trimer (57-59). Moreover, the SARS-CoV-2 Spike protein can exist in closed and open conformations, depending on whether the receptor-binding domains are buried in the protein or facing up towards the cell membrane. Interestingly, these dynamics are influenced by pH, with one or more receptor-binding domains facing up at neutral pH, while all of them facing down towards the protein at pH 4.5 (60), preventing binding of neutralizing antibodies against the receptor-binding domain. The protonation of several aspartate residues in a hinge region seems to play a role in this conformational change, but no cluster of aspartate residues is present in the SARS-CoV-2 spike.

Taken together, our results point to a role for the reovirus aspartic acid cluster not only as a potential pH-dependent molecular switch, but also as a requirement for stability of the $\sigma 1$ Head at the low gastric pH all enteric viruses are exposed to and for the integrity of the JAM-A binding site.

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FIGURES AND FIGURE LEGENDS

Figure 1:

A) Structural model and domain organisation of reovirus $\sigma 1$. The model was generated by superposition of overlapping published crystal structures (pdb codes 3s6x and 6gap)(21, 22). The trimeric protein is shown in cartoon representation and coloured according to chain. The constructs used in this study are colour coded according to their length (Tail-Body in light blue, Body-Head in green, and Head in orange).

B-E) Thermostability of T3D $\sigma 1$ Tail-Body (C), Body-Head (D) and Head (E) constructs in buffers of different pH, with GST as comparison (B). Melting temperatures are plotted as averages of three independent experiments, with error bars showing standard deviation. The range of pH of stomach and endosome are highlighted in light grey. For GST, no melting temperatures were obtained below pH 4.4 because the protein denatured upon mixing with the low pH buffers before starting the experiment.

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641 Figure 2:

642A) Thermostability of T3D σ1 wild-type (black) and D345N (red) mutant Head. The range of
643pH of stomach and endosome are highlighted in light grey. Data is an average of three
644independent experiments, error bars represent the associated standard deviation.

645B) Analytical size exclusion chromatography of wild-type (grey and black) and D345N mutant
646Head (light red and red). Percentage of monomeric T3D σ1 Head is depicted after integrating
647absorbance peaks from the chromatograms (Fig. S1). Measurements were carried out
648directly after buffer exchange (light colour) and after a 4-hr incubation at room temperature
649(darker colour).

650C) Location of the aspartic acid cluster in the σ1 Head. σ1 is shown in cartoon
651representation, with one monomer coloured yellow, one blue and one red. Figure is based on
652pdb entry2OJ5 (10).

653D) Organisation of the aspartic acid cluster in the T3D σ 1. The wild-type protein is shown in
 654stick representation and coloured yellow for one chain and blue for its clockwise
 655neighbouring monomer. The D345N mutant is coloured grey. The hydrogen bonds from
 656Asp345 to Asp346cw are shown as dashed lines for the wild-type (black) and D345N (grey)
 657structures. Figure is based on pdb entries 2OJ5 and 2OJ6 (10). Atomic distances between
 658residues were measured with Coot.

659E) Atomic distances between side chain oxygen and nitrogen atoms of Asp345 (wild-type) or
 660Asn345 (mutant) to their hydrogen bonding partners. Distances were averaged over the six
 661crystallographically independent monomers in the structures and given with their standard
 662deviations.

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671 **Figure 3:**

672A) Thermostability of T3D $\sigma 1$ wild-type (black) and D345N (red) mutant Body-Head
673construct. The range of pH of stomach and endosome are highlighted in light grey. Data is an
674average of three independent experiments, error bars represent the associated standard
675deviation.

676B) Analytical size exclusion chromatography of wild-type (grey and black) and D345N mutant
677Body-Head (light red and red). Percentage of monomeric T3D $\sigma 1$ Body-Head is indicated
678after integrating absorbance peaks from the chromatogram. Measurements were carried out
679before (light color) and after a 4-hour incubation time (dark color).

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Supplemental Table 1: Buffers used during DSF measurements.

pH	Buffer
1.4	0.4 M HCl/KCl, 150 mM NaCl
2.0, 2.4, 3.0	0.4 M Glycine/HCl, 150 mM NaCl
3.4	0.4 M Citric Acid/Sodium Citrate, 150 mMNaCl
4.0, 4.4	0.4 M NaAcetate, 150 mMNaCl
5.0, 5.4	0.4 M NaCitrate, 150 mMNaCl
6.0, 6.4	0.4 M NaCacodylate, 150 mMNaCl
7.0	0.4 M NaPhospate, 150 mMNaCl
7.4	0.4 M NaHEPES, 150 mMNaCl
8.0, 8.4	0.4 M Tris/HCl, 150 mMNaCl
9.0, 9.4	0.4 M NaBorate, 150 mMNaCl

Supplemental Table 2: Buffers used during analytical gel filtration experiments.

pH	Buffer
3.0	20 mMGlycine/HCl, 150 mMNaCl
5.0	20 mMNaCitrate, 150 mMNaCl
7.1	20 mMNaHEPES, 150 mMNaCl
8.6	20 mMTris/HCl, 150 mMNaCl

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696 **Figure S1:** Analytical size exclusion traces of T3D wild-type (black) and D345N (red) $\sigma 1$
697 Head samples at pH 3.0, 5.0, 7.1, and 8.6. Absorbance peaks corresponding to trimers and
698 monomers of T3D $\sigma 1$ Head were integrated in Unicorn and are highlighted by a grey
699 background.

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