The Adsorption Protein of Filamentous Phage fd: Assignment of Its Disulfide Bridges and Identification of the Domain Incorporated in the Coat†

Andreas Kremser and Ihab Rasched*

Fakultät für Biologie, Universität Konstanz, Universitätsstrasse 10, P.O. Box 5560, D-78434 Konstanz, Germany

Received May 4, 1994; Revised Manuscript Received July 20, 1994®

ABSTRACT: The mature adsorption protein (g3p) of filamentous phage fd consists of 406 amino acids. It contains eight cysteine residues in total. To determine the disulfide bond pattern, purified g3p was proteolytically digested, and the resulting peptides were separated using RP-HPLC. N-Terminal sequencing and mass spectrometry of cysteine-containing fragments showed that each cysteine is involved in an intramolecular disulfide bond. The cystine sites are Cys7−Cys36, Cys46−Cys53, Cys188−Cys201, and Cys354−Cys371. In the native conformation of g3p, none of the disulfide bridges is accessible to alkylating agents after treatment with DTT. The cystine sites seem therefore to be located in the interior of the folded molecule or are shielded by components of the phage envelope. The part of g3p which is incorporated in the phage coat and hence is inaccessible to proteolytic cleavage was identified after treatment of phage particles with subtilisin: Immunoblots performed with antibody directed against g3p revealed essentially one g3p fragment with an apparent molecular weight of approximately 17 000 which resisted the proteolytic attack. The amino terminus of this peptide was determined to be glycine 254. This amino acid position correlates with the carboxy-terminal end of the second glycine-rich region (amino acids 218−256) in the primary structure of g3p. The notion that the extended carboxy-terminal g3p fragment is indeed entirely buried within the phage envelope is further supported by the fact that only polyclonal antibodies raised against purified g3p are able to react with this peptide, whereas those against phage coat-associated g3p are not.

The filamentous phage fd belongs to a group of nonlytic bacteriophages that infect *Escherichia coli* cells carrying an F- episome (for reviews, see Rasched & Oberer, 1986; Model & Russel, 1988). Gene 3 protein[1] (g3p) located together with g6p in three to five copies at one end of the phage particle (Grant et al., 1981; Simons et al., 1981) is indispensable for the normal infection process. It mediates the adsorption of the phage to the tip of the host F-pili (Caro & Schnös, 1966; Pratt et al., 1969; Jacobson, 1972) and is also involved in penetration of the single-stranded phage DNA into the host cell (Stengele et al., 1990). As revealed by electron microscopy, g3p exhibits a "knob on stem" structure pinned to the tip of the phage filament. The knob can be released by treatment with subtilisin, resulting in noninfectious phage particles (Gray et al., 1981).

The primary structure of g3p was deduced from the sequence of the fd genome (Beck & Zink, 1981). The protein is synthesized as a precursor (424 amino acids) with an 18-residue signal sequence which is removed upon incorporation into the host inner membrane (Goldsmith & Konigsberg, 1977). Mature g3p therefore consists of 406 amino acids containing eight cysteines at positions 7, 36, 46, 53, 188, 201, 354, and 371. A defined carboxy-terminal sequence (amino acids 379 to 401) anchors the protein to the host membrane, whereas the long amino-terminal part including the cysteines protrudes into the periplasm (Boeke & Model, 1982; Davis et al., 1985). A remarkable feature of the primary structure of g3p are two glycine-rich sequences encompassing residues 68−86 and 218−256. They consist of monotone repeats of tetrapeptides (GGGS) and pentapeptides (EGGGS). The role of these regions has not yet been elucidated.

Several sections within the primary structure of g3p were correlated with its different functions by deletion analysis. For example, when residues 29−340 are deleted, incorporation into the phage coat is abolished and hence polyphage are generated (Crissman & Smith, 1984). Deletion of amino acids 29−197 results in loss of infectivity (Nelson et al., 1981). Finally, it was shown that residues 99−196 are part of the attachment site, whereas amino-terminal stretches are involved in the attachment to the host receptor as well as in the penetration of the phage DNA (Stengele et al., 1990).

In contrast to these detailed investigations concerning the linear organization of the functions of g3p, almost nothing is known about the folded structure of the polypeptide chain.

This study aims at gathering information about the higher order structure of g3p by investigating the presence of disulfide bridges and assigning their respective positions.

MATERIALS AND METHODS

Purification of g3p. Fd phages (5−10 mg/mL) were dissociated with 1% sodium dodecyl sulfate (SDS) at 37 °C for 20 min. DNA and coat proteins were separated by size-exclusion chromatography on a HiLoad Superdex 200 (26/60) column (Pharmacia). Elution was performed with 50

† This work was supported by Grant Ra 220/3-3 of the Deutsche Forschungsgemeinschaft.

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* October 15, 1994.

[1] Abbreviations: g3p, gene 3 protein; RP-HPLC, reversed-phase high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; Cys, half-cystine; PE-cysteine, (pyridylethyl)cysteine; SBD-F, ammonium 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; CH_3CN, acetonitrile; EDTA, ethylenediaminetetraacetic acid.

0006-2960/94/0433-13954$04.50/0

mM Na_2SO_4, 5 mM citric acid, 0.1% SDS (pH 3.0) at a flow rate of 50 mL/h. DNA and protein peaks were monitored at 280 nm. g3p-containing fractions were pooled and concentrated in Centricon 30 tubes (Amicon). For the initial digestion with thermolysin, the buffer was changed by washing the protein solution three times with 2 mL of 20 mM Tris/HCl (pH 7.9) in a Centricon 30 tube.

Thermolysin Digestion of Purified g3p. Thermolysin (Sigma) was dissolved in 20 mM $CaCl_2$. Digestion of g3p (0.8−1 mg) was performed in 20 mM Tris/HCl (pH 7.9) at 42 °C for 2.5 h at an enzyme to substrate ratio of 1.5 (w/w). After incubation, the digestion mixture was directly subjected to RP-HPLC separation.

Chymotrypsin Digestion of Thermolysin Fragments. Lyophilized HPLC fractions of the thermolysin digest were dissolved in 100 μL of 65 mM Tris/HCl (pH 7.8), 5 mM $CaCl_2$, 0.01% SDS. Then, 4 μg of chymotrypsin (Boehringer, sequencing grade) dissolved in 1 mM HCl was added, and the mixture was incubated at 37 °C for 3.5 h.

Subtilisin Digestion of Thermolysin Fragments. Selected cysteine-containing HPLC fractions of the thermolysin digest were lyophilized on a speedvac concentrator (Bachmann) and redissolved in 50 mM Tris/HCl (pH 8.0), 0.05% SDS. Subtilisin (Boehringer) was added at a molar ratio of approximately 1:50 (enzyme:peptide). After incubation at 37 °C for 5 h, the mixture was directly subjected to RP-HPLC.

Trypsin Digestion of Subtilisin Subfragments. The selected lyophilized HPLC fraction of the subtilisin digest was redissolved in 100 mM Tris/HCl (pH 8.0), 0.01% SDS. Next, 2.5 μg of trypsin (Boehringer; sequencing grade) in 0.01% trifluoroacetic acid (TFA) was added. The digestion was performed at 37 °C for 3 h.

RP-HPLC Purification and Separation. For purification of undigested g3p and separation of its thermolysin fragments, a Lichrospher 100 RP-8 (5 μm, Merck) column was used. The separation of peptides of other digests was performed on a Lichrospher 100 RP-18 (5 μm, Merck) column. Linear gradients were applied using 0.1% TFA (Merck; uvasol grade) in H_2O as solution A and 0.1% TFA/80% (or 70%) CH_3CN (Merck) as solution B. The column temperature was set to 40 °C. Protein and peptides were eluted at a flow rate of 0.6 or 0.7 mL/min and detected at 212 nm.

SBD-F Fluorometric Assay. Cysteine-containing HPLC fractions were detected by incubation of aliquots (20−60 μL) of each HPLC fraction with SBD-F and tributylphosphine according to Sueyoshi et al. (1985). The fluorescence intensities were measured in a Kontron SFM 25 or a Merck/Hitachi F-1050 fluorescence spectrometer with excitation at 385 nm and emission at 515 nm.

To examine the status of cysteines, the SBD-F assay was carried out under both reducing and nonreducing conditions. In nonreduced samples, tributylphosphine was replaced by an equal volume of *N,N*-dimethylacetamide (Aldrich). Further incubation conditions remained the same.

Alkylation of Protein and Peptides with 4-Vinylpyridine. HPLC-purified g3p was lyophilized and redissolved in 100 mM boric acid/NaOH (pH 8.7). Denaturation was performed with 1% SDS. To alkylate any free cysteine, 4-vinylpyridine (Serva) was added in an approximately 500-fold molar excess over total cysteines. Subsequently the mixture was incubated at 37 °C for 60 min. For pyridylethylation of total cysteines, g3p was reduced with dithiothreitol (DTT) at 50 °C for 50 min using a 100-fold molar excess over cysteines. In this case alkylation was performed with a 20-fold molar excess of 4-vinylpyridine over the total amount of thiol groups. The reaction mixtures were lyophilized, redissolved in 0.1% TFA, and subjected to RP-HPLC purification before mass spectrometry was performed.

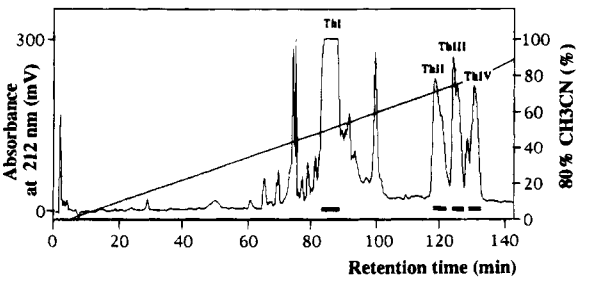

FIGURE 1: Partial separation of the thermolytic digest of g3p on a C8 column. Chromatographic conditions are as described in materials and methods. Pools corresponding to ThI, ThII, ThIII, and ThIV are indicated by bars.

Pyridylethylation of cysteines in g3p fragments and in phage coat-associated g3p was carried out using the same procedure. In the latter case the denaturation step was omitted.

N-Terminal Amino Acid Sequence Analysis and Mass Spectrometry. Automated amino acid sequence analysis was performed on a Model 477A pulsed-liquid protein sequencer (Applied Biosystems). Aliquots of selected HPLC fractions (60−150 μL) were directly applied on the polybrene-coated glasfiber membrane. Mass spectra of g3p fragments and g3p were recorded using a Vestec A201 quadrupol mass analyzer or a TSQ 700 Finigan MAT, Bremen, respectively.

Subtilisin Treatment of fd Phages. fd phages (5 mg/mL) were incubated with 100% subtilisin (weight enzyme per weight g3p) in 10 mM Tris/HCl, 1 mM EDTA (pH 7.5) at 37 °C for 60 min. Protease and released peptides were removed using cesium chloride gradient centrifugation (42 000 rpm; 22 h; 12 °C).

RESULTS

Purified g3p was first digested with thermolysin. The small amount of protease we used together with the short cleavage period on the one hand resulted in incomplete digestion of g3p but on the other hand was necessary to minimize the possibility of disulfide exchange reactions. After partial separation of the cleavage products (Figure 1), aliquots of each HPLC fraction were screened for fluorescence using the SBD-F assay described in materials and methods. Fluorescent and therefore cystinyl peptide-containing fractions were pooled as indicated in Figure 1.

Identification of Cystine Site Cys7−Cys36. The pooled fractions designated as ThI (Figure 1) were further digested with subtilisin. The resulting peptide mixture was separated by C18-HPLC (Figure 2), and again the fluorescence of each fraction was assayed (data not shown). The peak at 54 min (Figure 2) was strongly fluorescent but contained more than one peptide as revealed by N-terminal sequencing. This peak was further treated with trypsin. After C18 separation of this digest (chromatogram not shown), a fraction was detected at 58 min that exhibited strong fluorescence in the SBD-F assay under reducing conditions. Edman degradation showed that only one fragment starting with the original

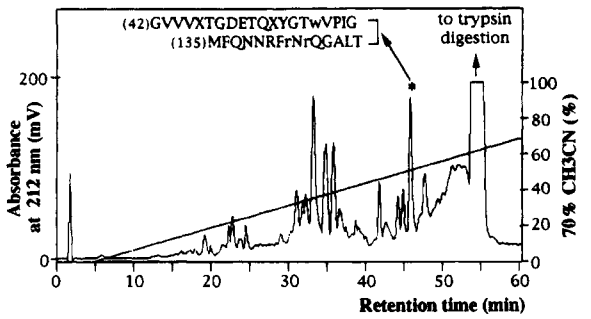

FIGURE 2: Subtilisin digest of ThI (Figure 1) analyzed by C18-HPLC. The peak at 46 min (marked with an asterisk) contained two internal fragments of g3p. The sequences of these peptides are shown. X denotes a cysteine residue expected from the known sequence but cannot be detected. Tentatively assigned amino acids are given in lower case letters. The peak at 54 min was further digested with trypsin.

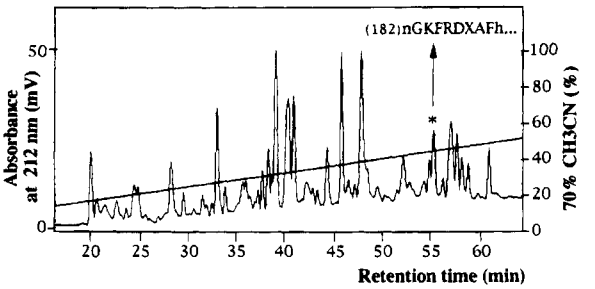

FIGURE 3: C18-HPLC chromatogram of the chymotrypsin digest of ThII (Figure 1). The g3p fragment with the sequence indicated was the only peptide present in the peak at 55 min (marked with an asterisk). X denotes cysteine 188 which cannot be detected. Tentatively assigned amino acids are given in lower case letters.

N-terminus of g3p was present in this fraction. The mass of this fragment was determined as 4548 Da, indicating that it consists of the first 39 amino acids of g3p and thus includes cysteines 7 and 36. Performing the SBD-F assay under nonreducing conditions demonstrated that these two cysteines are disulfide-bonded. In contrast to the reducing assay, no fluorescence could be detected. Interestingly, the three potential cleavage sites for trypsin present in this fragment all resisted the protease. This may be the consequence of a "folded" structure of this peptide due to the presence of the disulfide bridge which makes the target peptide bonds inaccessible to trypsin.

Identification of Cystine Site Cys46–Cys53. N-Terminal sequencing of the strongly fluorescent HPLC fraction indicated with an asterisk in Figure 2 showed that this peak which eluted at 46 min was composed of two peptides derived from the interior sequence of g3p. The major peptide started with glycine 42 and included cysteines 46 and 53. Its mass was determined as 2195 Da, which corresponds to a length of 21 amino acids. The coeluted second peptide started with methionine 135 and ended after 15 cycles with threonine 149. The correctness of this size was further supported by the observed mass of 1855 Da for this peptide (calculated mass: 1854 Da). No cysteine is included in this g3p fragment.

To decide whether cysteines 46 and 53 are disulfide-bonded, an aliquot of the relevant HPLC fraction was treated with DTT and vinylpyridine, while another aliquot was incubated with the alkylating agent only. Both samples were subjected to Edman degradation. While the reduced sample showed a clear PE-cysteine peak at positions 5 and 12 of the peptide, no PE-cysteine signal could be detected in the corresponding cycles of the nonreduced sample. Furthermore fluorescence in the SBD-F assay could only be observed under reducing conditions. These results clearly demonstrate that cysteines 46 and 53 form a disulfide bridge.

Identification of Cystine Site Cys188–Cys201. The pooled HPLC fractions designated as ThII in Figure 1 were further digested with chymotrypsin and subsequently analyzed by C18-HPLC (Figure 3). The fluorescent fraction at 55 min (indicated with an asterisk in Figure 3) contained a g3p-fragment starting with asparagine 182 as determined by N-terminal sequencing. Mass analysis of this fraction gave a value of 2581 Da for this peptide, corresponding to a length of 22 amino acids. It thus includes cysteines 188 and 201.

Table 1: Predicted and Determined Masses for g3p Treated As Indicated

g3p[a]	calculated mass	observed mass
untreated	42566	42563
+DTT/+VP	43407	43410
+VP		42565

[a] DTT, dithiothreitol; VP, 4-vinylpyridine (incubation conditions are described in Materials and Methods).

Since the SBD-F assay of this HPLC fraction was positive only under reducing conditions, cysteine 188 and cysteine 201 are also involved in a disulfide bond.

Determination of the Total Disulfide Content of g3p. A peptide containing cysteine 354 and/or cysteine 371 could be detected neither in the fluorescent fraction ThIII nor in ThIV (Figure 1) nor in other subdigests. Since the carboxy-terminal part of g3p, which includes the anchor sequence, predominantly contains hydrophobic amino acids, the problem might be that peptides derived from this part of the polypeptide chain aggregate under the conditions used. They may have been lost during the incubation, lyophilization, and purification steps.

In order to determine the status of cysteines 354 and 371 we investigated the total disulfide content of g3p. For this purpose g3p isolated by size-exclusion chromatography was further purified by C8-HPLC. The protein was lyophilized and redissolved in buffer containing 1% SDS for complete denaturation. This solution was divided into three parts. The first part was reduced with DTT and afterwards incubated with vinylpyridine to alkylate cysteines quantitatively. The second one was directly incubated with vinylpyridine to pyridylethylate any free cysteine. The third one remained untreated. All three samples were repurified using C8-HPLC and subjected to mass spectral analysis. The results are shown in Table 1. The observed mass of 42 563 Da for the untreated g3p exactly correlates with the molecular weight calculated from the amino acid sequence. The mass of g3p pyridylethylated under reducing conditions was found to be 43 410 Da. As the molecular weight of PE-cysteine is 105 higher than that of non-derivatized cysteine, the difference of 840 clearly demonstrates that each of the eight cysteines has been alkylated and was therefore accessible to vinylpyridine. g3p incubated with the alkylating agent only exhibited the same molecular weight as the untreated protein, indicating that there are no free cysteines in g3p. Thus cysteine 354 and cysteine 371 must be disulfide-bonded.

Accessibility of the Disulfide Bridges to Reducing and Alkylating Agents in Native, Phage Coat-Associated g3p. To

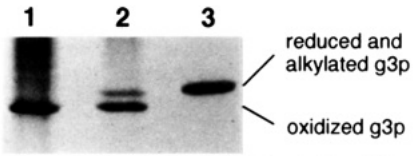

FIGURE 4: Nonreducing SDS−PAGE. A 10−15% gradient gel (Phast Gel; Pharmacia) was used to visualize the differences in the electrophoretic mobility of reduced and oxidized g3p. The gel was silver-stained. Lane 1: fd phages. Lane 2: fd phages treated with DTT and vinylpyridine. After treatment the phage particles were washed thoroughly with 10 mM Tris/HCl (pH 7.0) using a Centricon 30 tube to eliminate the agents quantitatively. Lane 3: fd phages first dissociated with 1% SDS and subsequently treated in the same way as described for lane 2.

examine the accessibility of the disulfide bridges of native g3p to reducing and alkylating agents, fd phages were first treated with DTT and subsequently with vinylpyridine. After the incubation steps, the fd solution was thoroughly washed to eliminate the reagents quantitatively. As a control, fd phages were first dissociated with SDS and then treated in the same way. Both samples were subjected to nonreducing SDS−PAGE (Figure 4).

As shown in Figure 4, there is no difference in the electrophoretic mobility of g3p of untreated phages (lane 1) compared with the g3p of phages incubated with DTT and vinylpyridine (lane 2). This indicates that in both cases all cysteine residues are present in the oxidized form. The reduced and alkylated protein (lane 3) exhibits a lower mobility because of its more extended shape. These findings demonstrate that even when treated with DTT, the cystine sites of native, phage-integrated g3p are inaccessible to the alkylating agent. Thus the disulfide bridges seem to be buried in the interior of the molecule or are protected by components of the phage envelope. Since in general only5−10% of the total amount of fd phages are infectious, we propose that the reproducible appearance of the minor band of reduced g3p in lane 2 (Figure 4) results from that fraction of damaged phages which have no properly folded g3p.

Identifying the Part of g3p Incorporated in the Phage Coat. g3p is accessible to subtilisin digestion as was previously shown by Gray et al. (1981) and Armstrong et al. (1981). We therefore treated fd phages with subtilisin in a ratio of 1:1 with g3p (weight:weight) to remove the exposed part of g3p. With the amount of protease used, only negligible cleavage of the major coat protein occurs (Schwind et al., 1992). The integrity of the phage coat is therefore not affected. After the proteolytic attack the phages were repurified by cesium chloride gradient centrifugation. Phage particles were disrupted by boiling in sample buffer, and the coat proteins were separated by tricine−SDS−PAGE (Schägger & von Jagow, 1987). Immunoblots were performed with either polyclonal anti-g3p antibody or polyclonal anti-fd antibody (Figure 5). Lane 3 (Figure 5) shows the situation after subtilisin treatment of the phage particles but before their purification by centrifugation. In this case several bands are detected with anti-g3p antibody, whereas after purification (lane 5) only one distinct and one faint and diffuse (slightly slower) band are seen, both with an apparent molecular weight of approximately $17-18 \times 10^3$. Although polyclonal antibodies directed against whole fd phages in principle are able to recognize both full size g3p and g3p fragments (lanes 2 and 4, respectively), they fail to react with the 17 kDa g3p fragments in subtilisin treated phages

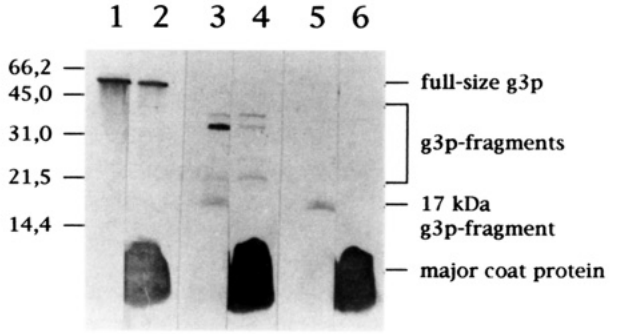

FIGURE 5: Immunoblot of tricine−SDS−PAGE. Molecular weight markers (kDa) are indicated on the left side. Lanes 1, 3, and 5 were incubated with polyclonal anti-g3p antibody. Lanes 2, 4, and 6 were incubated with polyclonal antibody directed against whole fd phages. Lanes 1 and 2: fd phages. Lanes 3 and 4: fd phages treated with subtilisin. Lanes 5 and 6: fd phages repurified on a cesium chloride gradient after treatment with subtilisin.

(lanes 4 and 6). This fact as well as the fact that the 17 kDa fragments cosediment with the phage particle in a cesium chloride gradient demonstrate that this domain of g3p is inaccessible both to the immune response machinery of the rabbit and to subtilisin, strongly suggesting that this polypeptide fragment is buried within the phage envelope.

The 17 kDa bands were blotted onto a PVDF membrane and subjected to automatic Edman degradation. Only one N-terminal sequence for the peptides beginning with glycine 254 could be obtained. This amino acid position correlates with the carboxy-terminal end of the second glycine-rich region (amino acids 218−256) in the primary structure of g3p.

DISCUSSION

The presented investigations are the first attempt to gather information about the structural organization of fd−g3p; we examined the presence of disulfide bridges in this protein. The data demonstrate that each of the eight cysteines of mature fd−g3p is involved in a disulfide bond. The cystine sites are Cys7−Cys36, Cys46−Cys53, Cys188−Cys201, and Cys354−Cys371.

Six of the eight cysteine positions (cysteines 7, 36, 46, 53, 354, and 371) are almost completely conserved in homologous sequences of the adsorption protein of the related filamentous phage IKe (Peeters et al., 1985; Bross et al., 1988). We therefore suggest that IKe−g3p displays the same disulfide pattern as fd−g3p.

In a previous publication we reported that the functions of fd−g3p in adsorption and penetration are associated with different regions of its N-terminal amino acid sequence (Stengele et al., 1990). For morphogenesis and correct assembly to unit-length phages, a C-terminal section of g3p is necessary (Crissman & Smith, 1984). These and other observations (Armstrong et al., 1981) led to the conclusion that g3p is made up of functionally independent domains. Our finding that disulfide bridges are always formed between two successive cysteines indicates that the adsorption protein is composed of "local" folds which might represent the structural basis for the observed modular organization into functional domains. The fact that an N-terminal peptide (the first 39 amino acids) containing cystine site Cys7−Cys36 survives successive digestions with the proteases thermolysin, subtilisin, and trypsin strongly supports this suggestion. It is likely that this peptide has a compact "folded" conforma-

tion of its own. The formation of these "local" folds seems to be a prerequisite for the native trypsin-resistant conformation of g3p. For example, g3p mutants, which contain a cysteine to serine substitution at position 7, are strongly impaired in adsorption to the host cell. Furthermore these mutants are readily digested by trypsin in contrast to wildtype g3p, indicating the loss of the native conformation (Rampf, 1994).

Here we show that all disulfide bridges of g3p are inaccessible to the alkylating agent vinylpyridine after treatment with DTT. The cystine sites seem therefore to be buried in the interior of the protein or are protected by components of the phage coat. This finding may explain the observation of Vaccaro et al. (1987) that phage fd infectivity is not affected by reducing agents.

After subtilisin treatment of whole phages a 17 kDa g3p fragment starting with glycine 254 was identified which is detectable only with anti-g3p antibody but not with anti-fd antibody. These data suggest that the 17 kDa peptide is entirely buried within the phage envelope. Crissman and Smith (1984) showed that a g3p mutant in which amino acids 29−340 are deleted is no longer assembled into the phage coat. As a consequence only polyphages are produced. In contrast to this mutant, phage fKN16 in which amino acids 29−197 of wild-type g3p are removed generates unit-length phages with the truncated adsorption protein incorporated in the phage envelope. From these observations it was concluded that the morphogenetic function of g3p is located somewhere between amino acids 198 and 406 (Crissman & Smith, 1984). As mentioned above, the extended carboxy-terminal part of g3p beginning with amino acid position 254 is incorporated in the phage coat. Accordingly, we can further restrict the morphogenetic activity of g3p to its last 150 amino acids. Since g3p mutants in which either cysteine 354 or cysteine 371 is replaced by serine are not assembled (unpublished results), we furthermore suggest that this cystine site stabilizes a carboxy-terminal functional domain necessary for morphogenesis. Whether the morphogenetic activity of the last 150 amino acids of g3p depends on its oligomerization with g6p (the second minor coat protein located at the g3p-end of the phage; Gailus & Rasched, 1994), on homo-oligomerization of g3p (Glaser-Wuttke et al., 1989) or on an interaction with the major coat protein remains to be determined.

The primary structure of g3p bears two glycine-rich stretches (amino acids 68−86 and 218−256). It is remarkable that the protease accessible part of g3p starts with the second one (amino acid 253; counting from the N-terminus). The peptide bond upstream of glycine 254 obviously represents the ultimate target for the subtilisin attack: the polypeptide beginning with this residue accumulates to amounts detectable by sequence analysis. The faint, diffuse 17 kDa band in Figure 5 (lanes 3 and 5) for which no defined N-terminal sequence could be determined in our opinion represents various g3p-fragments generated within the glycine-rich section and differing in length only by few amino acids. We propose that "incomplete digestion" of native g3p in this case is very probably due to a mobility of the repetitive glycine-rich sequence which may cause different accessibilities of the peptide bonds to the unspecific protease subtilisin in this motive. Thus both of the glycine-rich sequences in g3p may function as hinge regions to maintain the flexibility between the exposed functional domains of g3p.

ACKNOWLEDGMENT

We wish to thank Drs. Przybylski and Hucho for providing the MS facilities, Drs. Glocker and Franke for recording the mass spectra of g3p fragments and full-size g3p, respectively, and L. Cobianchi for excellent technical assistance.

REFERENCES

Armstrong, J., Perham, R. N., & Walker, J. E. (1981) *FEBS Lett. 135*, 167−172.
Beck, E., & Zink, B. (1981) *Gene 16*, 35−58.
Boeke, J. D., & Model, P. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5200−5204.
Bross, P., Bußmann, K., Keppner, W., & Rasched, I. (1988) *J. Gen. Microbiol. 134*, 461−471.
Caro. L., & Schnös, M. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 126−132.
Crissman, J. W., & Smith, G. P. (1984) *Virology 132*, 445−455.
Davis, N. G., Boeke, J. D., & Model, P. (1985) *J. Mol. Biol. 181*, 111−121.
Gailus, V., & Rasched, I. (1994) *Eur. J. Biochem. 222*, 927−931.
Glaser-Wuttke, G., Keppner, J., & Rasched, I. (1989) *Biochim. Biophys. Acta 985*, 239−247.
Goldsmith, M., & Konigsberg, W. H. (1977) *Biochemistry 16*, 2686−2694.
Grant, R., Lin, T. C., Konigsberg, W. H., & Webster, R. E. (1981) *J. Biol. Chem. 256*, 539−546.
Gray, C. W., Brown, R., & Marvin, D. A. (1981) *J. Mol. Biol. 146*, 621−627.
Jacobson, A. (1972) *J. Virol. 10*, 835−843.
Model, P., & Russel, M. (1988) In *The Bacteriophages* (Calendar, R., Ed.), Vol. 2, pp 375−456, Plenum Press, New York.
Nelson, K., Friedmann, S., & Smith, G. P. (1981) *Virology 108*, 338−350.
Peeters, B. P. H., Peters, R., Schoenmakers, J. G. G., & Konings, R. N. H. (1985) *J. Mol. Biol. 181*, 27−39.
Pratt, D., Tzagoloff, H., & Beaudoin, J. (1969) *Virology 39*, 42−53.
Rampf, B. (1994) Dissertation, Konstanz.
Rasched, I., & Oberer, E. (1986) *Microbiol. Rev. 50*, 401−427.
Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
Schwind, P., Kramer, H., Kremser, A., Ramsberger, U., & Rasched, I. (1992) *Eur. J. Biochem. 210*, 431−436.
Simons, G. F. M., Konings, R. N. H., Schoenmakers, J. G. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4194−4198.
Stengele, I., Bross, P., Garcés, X., Giray, J., & Rasched, I. (1990) *J. Mol. Biol. 212*, 143−149.
Sueyoshi, T., Miyata, T., Iwanaga, S., Toyo'oka, T., & Imai, K. (1985) *J. Biochem. 97*, 1811−1813.
Vaccaro, M., Boehler-Kohler, B., Müller, W., & Rasched, I. (1987) *Biochim. Biophys. Acta 923*, 29−34.