Effects of guanidinium hydrochloride on the structure and immunological properties of *Bordetella pertussis* fimbriae

Alexandra M. PEARCE,* Laurence I. IRONS,† Andrew ROBINSON† and Richard N. SEABROOK*‡
Divisions of *Biotechnology and †Biologics, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, U.K.

Denaturation of *Bordetella pertussis* fimbrial preparations by guanidinium hydrochloride (GdnHCl) has been characterized using static light scattering, c.d., fluorescence and antibody recognition. The susceptibility of Fim2+3 (a mixed preparation of two fimbrial types) to GdnHCl was found to be highly dependent on pH; as the pH was increased from pH 7.2 to 10.5, the concentration of GdnHCl required to induce 50% denaturation was decreased. At pH 10.5, Fim2+3 was denatured by GdnHCl in a three-step pathway comprising: (1) formation of a pre-denaturation intermediate at < 1.0 M-GdnHCl; (2) dissociation of the fimbrial polymer into subunits between 2 M- and 3.2 M-GdnHCl; and (3) subunit unfolding between 2.8 M- and 3.6 M-GdnHCl. A similar pathway was also found for the denaturation of the individual fimbrial types, Fim2 and Fim3, except that unfolding of either subunit commenced at a lower GdnHCl concentration (2.2 M) than that found for the mixture of fimbriae, Fim2+3. The second step in the denaturation pathway, dissociation into subunits, was partially reversible, but the renaturation and reassociation of fully unfolded subunits to form fimbria-like structures was not achieved. These findings demonstrate that the GdnHCl denaturation of complex polymeric proteins is unlikely to follow a reversible two-state denaturation pathway, and support the involvement of a chaperone-like protein in the folding and assembly of the fimbriae *in vivo*. Measurement of the ability of anti-fimbrial monoclonal antibodies to recognize intermediates in the denaturation pathway enabled the identification of two types of epitope which were dependent on different aspects of fimbrial tertiary/quaternary structure.

**INTRODUCTION**

*Bordetella pertussis*, the aetiological agent of whooping cough, can express two antigenically distinct fimbriae, Fim2 and Fim3 (Robinson et al., 1989a). Both proteins are assembled from their respective monomer into long (∼100 nm) polymeric helical structures (Steven et al., 1986) which extend from the bacterial outer membrane and are considered to be involved in the attachment of the bacterium to the respiratory tract (Gorringe et al., 1985; Robinson et al., 1989b). The monomeric subunits of Fim2 and Fim3 have molecular masses of 22.5 kDa and 22.0 kDa respectively and have been cloned (Lively et al., 1987; Mooi et al., 1990). The DNA-deduced amino acid sequences of the two monomers indicate that the subunits share a high degree of amino acid identity (Mooi et al., 1990), which suggests that the proteins may also possess a similar three-dimensional structure. A mixed preparation of Fim2 and Fim3 (Fim2+3), purified from a Fim2+ strain, Fim3 strain, constitutes a major component of an acellular vaccine which has recently completed a phase-II clinical trial (Miller et al., 1991).

In order to further characterize the structure and conformation of the fimbriae, we have studied the guanidinium hydrochloride (GdnHCl) denaturation pathway of the mixed fimbrial preparation, Fim2+3, and compared the results obtained with those from the GdnHCl denaturation of the individual Fim2 and Fim3 proteins (which are purified in lower yields). Most other protein denaturation and refolding studies have been carried out on small monomeric and oligomeric proteins which frequently follow a two-state denaturation pathway (Creighton, 1978; Pace, 1986). In contrast, *B. pertussis* fimbriae are complex polymeric proteins which at physiological pH have been found to form bundles composed of individually aligned fimbrial polymers (Steven et al., 1986). Furthermore, by assessing the ability of antibodies to recognize the intermediates produced during the denaturation of the fimbriae, different types of fimbrial epitope have been identified.

A preliminary account of part of this work was presented as a communication at the Sixth International Symposium on Pertussis (Robinson et al., 1990).

**EXPERIMENTAL**

**Materials**

GdnHCl (99 % pure) and antibody–enzyme conjugates were purchased from Sigma; NAP-5 and PD-10 G-25 Sephadex columns from Pharmacia; newborn calf serum from Gibco, and Tween 20 and 3,3,5,5-tetramethylbenzidine from Aldrich Chemical Co.

**Bacterial strains and growth**

*B. pertussis* strain Wellcome 28 was obtained from Dr. P. Novotny (Wellcome, Beckenham, Kent, U.K.), strain Tohama from Dr. C. Manclark (NIH, Bethesda, MD, U.S.A.), and strain 10907 from NCTC. Strains were stored as freeze-dried suspensions and recovered by growth for 48 h at 35 °C on charcoal agar containing 10% (v/v) defibrinated horse blood. The growth from plates was inoculated into CL medium containing 2,6-O-dimethyl-β-cyclodextrin (Imazumii et al., 1983) and incubated with shaking for 24 h at 35 °C. A 10 ml portion of this primary culture was used to inoculate 300 ml of CL medium in 2.5 litre Thompson bottles and incubated at 35 °C with shaking for 48 h. The bacteria were harvested by centrifugation (5000 g, 30 min) and washed in water.

**Purification of fimbriae**

(a) Fim2+3. The Fim2+3 mixture (approximate ratio of 3:2) was isolated from *B. pertussis* strain Wellcome 28, serotype 1. F2,3 (Robinson et al., 1989a) by homogenization of the cells

Abbreviations used: Fim2+3, mixed fimbrial preparation; GdnHCl, guanidinium hydrochloride; McAb, monoclonal antibody.

* To whom correspondence should be addressed, at: Division of Biotechnology, PHLS, CAMR, Porton Down, Salisbury SP4 0JG, U.K.
and repeated ammonium sulphate precipitation of the extract as previously described for Agglutinogen 2 (Robinson et al., 1989b).

(b) Separate Fim2 and Fim3. Fim2 was purified from *B. pertussis* strain Tohama (serotype F2) by the urea extraction procedure described for Agglutinogen 3 (Robinson et al., 1989b), except that the dialysis against 0.01 M-Tris/0.1 M-MgCl₂, pH 7.0, was repeated 2–3 times. Similarly, Fim3 was isolated from *B. pertussis* strain 10907 (serotype F3).

All isolated fimbriae preparations were clarified by centrifugation at 30000 g for 2 h and were >95% pure on SDS/PAGE. Protein concentration was estimated by an automated Lowry procedure.

**Denaturation, reassociation and renaturation**

Protein solutions (200–400 μg/ml) were transferred into either 0.05 M-phosphate/0.1 M-NaCl, pH 7.2, or 0.125 M-ethanol-amine/0.1 M-NaCl, pH 10.5, containing GdnHCl, by buffer exchange on G-25 Sephadex columns. The material was incubated overnight at 4 °C to ensure equilibration. Renaturation/reassociation was attempted by first diluting an aliquot of the denatured sample to 10–200 μg of protein/ml in the denaturing buffer, followed by either dialysis or dilution into the buffer(s) used for renaturation. Several different conditions were employed for renaturation which varied in either pH or temperature, or in the concentration of GdnHCl, urea or protein employed.

**Spectroscopy**

Aliquots of the fimbriae exposed to different concentrations of GdnHCl were analysed for denaturation by fluorescence, c.d. and light scattering. Fluorescence measurements were made with a Perkin–Elmer LS-5 spectrofluorimeter, on 20–40 μg of protein/ml in a 1 cm pathlength quartz cuvette maintained at 30 °C. Tyrosine fluorescence was detected at 306 nm using a 280 nm excitation wavelength. Bandwidths of 5.0 nm and 2.5 nm were used for emission and excitation wavelengths respectively.

Static light scattering measurements were made on fimbrial aliquots (200 μg of protein/ml; 0.22 μm-filtered) using a Malvern K7032 photon correlator. The 488 nm wavelength source was provided by a Spectra Physics Argon ion laser, and scattered light was detected as counts/s with a 10 nm (100 Å) aperture photodetector at 90° to the incident light.

C.d. spectra were measured with a Jasco J-600 spectropolarimeter calibrated with 0.6% (w/v) ammonium d-10-camphorsulphanilum (Takakawa et al., 1985). Spectra were measured in the near-u.v. (260–320 nm) with a 1.0 cm pathlength cell. A 0.02 cm cell was used for far-u.v. scans (190–250 nm) of fimbriae which did not contain GdnHCl. For fimbriae in GdnHCl, far-u.v. scans were made in a 0.2 cm cell over the limited wavelength range of 220–250 nm (which was used because GdnHCl absorbs strongly at low wavelengths). Each spectrum was the average of six scans obtained with a time constant of 4 s and a 10 nm/min scan speed. A similarly averaged buffer blank spectrum was subtracted from the sample spectrum and the corrected spectrum converted to mean residue ellipticities using a mean residue weight of 107. In order to increase the precision of the measurements, GdnHCl-induced transition curves were also measured in a 0.2 cm cell at a fixed wavelength of 225 nm, with ellipticities collected every 1 s over a 5 min period. After deduction of a solvent blank value from each measurement, data were averaged and converted to mean residue ellipticities.

**Immunization**

Male CAMR (NIH) mice were immunized intraperitoneally with 0.5 ml of phosphate/NaCl buffer, pH 7.2, containing 5–25 μg of protein/ml adsorbed on to alhydrogel adjuvant. Animals were boosted with the same dose of immunogen after 3 weeks; sera was collected 6 weeks after the initial immunization and stored at -20 °C.

**Antibody recognition**

For antibody discrimination of native and non-native fimbrial structures, a solid-phase antigen e.l.i.s.a. was employed. By this procedure, each protein sample was diluted to 2.0 μg/ml in the appropriate GdnHCl-containing buffer and 100 μl was added to each well of a Nunc Immuno 1 microtitre plate. Following incubation overnight, the wells were washed with 3 × 100 μl of wash buffer [phosphate-buffered saline, pH 7.2, containing 0.1% (v/v) Tween], incubated for 2 h with 100 μl of antibody diluted in diluent (wash buffer containing 10% (v/v) newborn calf serum) and washed again. The wells were then incubated for 2 h with horseradish peroxidase conjugated to anti-(mouse IgG or IgM) in diluent. After washing, colour was developed by incubation for 20 min in 3,3,5,5-tetramethylbenzidine (0.01 mg/ml) in 0.012% (v/v) H₂O₂/0.05 M-acetate (pH 6.0). The enzyme reaction was stopped by addition of 35 μl of 5% (v/v) sulphuric acid and the absorbance was measured at 450 nm on a Titertek Multiscan.

Antibodies were employed either as a serial dilution to enable calculation of the end-point titre, which was defined as the antibody dilution that gave an absorbance at 450 nm of 1.0, or at a predetermined dilution that would yield an absorbance of between 1.0 and 2.0 with the native protein as antigen. This dilution of antibody ensured that the antibody concentration was limiting in the assay. The monoclonal antibodies (McAbs) 51/21, 51/24, Agg2A (directed against Fim2) and Agg3A and Agg3B (directed against Fim3) were each used as either cell supernatants or ascites fluids, and their preparation has previously been described by Ashworth et al. (1986). The polyclonal antipeptide serum anti-18A was raised in rabbits to a highly conserved amino acid sequence present in both Fim2 and Fim3 (Ashworth et al., 1989).

**RESULTS AND DISCUSSION**

**Fim2 + 3 c.d. spectra**

We have previously shown that Fim2 + 3 is stable to 6.0 M-GdnHCl in 0.05 M-phosphate/0.1 M-NaCl, pH 7.2, buffer (Robinson et al., 1990). However, in this study we have found that at pH 10.5 the fimbriae can be denatured at GdnHCl concentrations of <6.0 M (see below). To assess whether the change in pH had induced a conformational modification which had decreased the stability of the fimbriae to GdnHCl, c.d. spectra of Fim2 + 3 in the absence of GdnHCl at pH 7.2 and pH 10.5 have been compared.

In pH 7.2 buffer, the near-u.v. spectrum (Fig. 1a) showed a broad positive Cotton effect with a maximum at about 275 nm, a shoulder at 282 nm and a featureless negative band from 285 to 320 nm which has a minimum at 298 nm. In view of the absence of tryptophan residues in the fimbriae (Lively et al., 1987; Mooi et al., 1990), the 260–285 nm positive Cotton effect probably arises from the effect of tertiary folding on the tyrosine residues. The negative band extending from 285 nm to 320 nm may be attributed to the chirality of the cystine disulphide bond (Bayley, 1980). Except for a decrease in the intensity of the negative band, the Fim2 + 3 spectrum obtained at pH 10.5 was effectively identical to the pH 7.2 spectrum (Fig. 1a), indicating that the tertiary structure of the fimbriae was not significantly changed over this pH range.

The far-u.v. spectrum obtained at either pH 7.2 or pH 10.5 possessed weak positive Cotton effects with maxima at about 197 nm and 225 nm, and also a negative band with a minimum at about 205 nm (Fig. 1b). The similarity of the pH 7.2 and

1992

A. M. Pearce and others
**Bordetella pertussis fimbriae**

Vol. 283

**Fig. 1.** C.d. spectra of Fim2+3 at pH 7.2 and pH 10.5

(a) Near-u.v. and (b) far-u.v. spectra of 570 μg of Fim2+3/ml in 0.05 M-phosphate/0.1 M-NaCl, pH 7.2 and 0.125 M-ethanolamine/0.1 M-NaCl, pH 10.5, respectively.

**Fig. 2.** Effect of pH on the midpoint of the GdnHCl-induced unfolding of Fim2+3 as determined by c.d. spectroscopy

Fimbriae were unfolded in GdnHCl-containing buffers (0.05 M-phosphate/0.1 M-NaCl, pH 7.2; 0.05 M-glycine/0.1 M-NaCl, pH 8.9; and 0.125 M-ethanolamine/0.1 M-NaCl, pH range 9.5-10.5) by dialysis overnight (4 °C), and c.d. measurements made on aliquots of 200-300 μg of protein/ml.

**Fig. 3.** Fluorescence and c.d. of Fim2+3 in GdnHCl, pH 10.5

Following an overnight (4 °C) incubation of fimbriae in the indicated concentration of GdnHCl, aliquots were analysed in (a) for tyrosine fluorescence (▲) at 20 μg/ml and c.d. (■) at 200 μg/ml. Near-u.v. c.d. spectra shown in (b) are for 450 μg of Fim2+3/ml in 0, 0.3 and 0.85 M-GdnHCl.

The positive Cotton effect with a maximum at 225 nm is an unusual feature for protein c.d. spectra, and was found not to result from light-scattering artefacts associated with the large quaternary structure of the fimbriae (results not shown) (Tinoco et al., 1983). For other proteins and peptides a positive band between 220 and 230 nm has been attributed to either cysteine, in proteins with a low α-helix content (Hider et al., 1988; Romero et al., 1989), or tyrosine, or certain types of β-turn (Crisma et al., 1984). In the case of Fim2+3 we favour one of the latter two possibilities, since the protein has a low cystine content and treatment of the fimbriae with 0.02 M-dithiothreitol did not decrease the positive band at 225 nm, whereas it was abolished by GdnHCl (see below). Analysis of protein secondary structure content was not carried out because of the possible contribution of tyrosine residues to the far-u.v. spectrum.

**pH-dependence of GdnHCl denaturation**

As the pH of the denaturing buffer was increased from pH 7.2, the midpoint of the Fim2+3 denaturation transition was found to occur at successively lower GdnHCl concentrations (Fig. 2). This change in stability to GdnHCl does not appear to be the result of a major conformational change in protein structure (discussed above) but could, however, result from either the effect of pH on the ionization of a critical amino acid(s) side-chain, or enhanced solubility of the fimbriae due to debundling of their aggregated state (Steven et al., 1986). The apparent

pH 10.5 far-u.v. spectra confirms that there was no major modification of protein secondary structure on changing from pH 7.2 to pH 10.5. The shape of the far-u.v. spectra and the low intensity of the bands (Fig. 1b) suggests that there is little α-helix or β-sheet contribution to the secondary structure of the fimbriae.
stability of pertussis fimbriae to GdnHCl at neutral pH is similar to that found for Escherichia coli type 1 pili (Eschdat et al., 1981), which required 8.3 M-GdnHCl at pH 8.0 for dissociation.

**Fim2 + 3 denaturation at pH 10.5**

As the GdnHCl concentration was increased at pH 10.5, the fimbriae displayed a three-step GdnHCl denaturation pathway comprising: (a) a pre-denaturational intermediate; (b) dissociation of the fimbriae to yield monomeric subunits; and (c) subunit unfolding.

(a) **Pre-denaturational transition.** At concentrations of GdnHCl between zero and 1.0 M, there was a minor transition in fimbrial structure which was detectable by c.d., fluorescence, light scattering and McAb recognition (see Figs. 3, 4 and 6). This suggests that, at low concentrations (< 1.0 M), GdnHCl induces the formation of a relatively stable pre-denaturational intermediate. Similar intermediates have been identified for other proteins, but the relationship of these structures to the denaturation pathway is uncertain (Creighton, 1978). In some instances the intermediates are the result of GdnHCl interacting with a specific protein domain (Bismuto & Irace, 1988). However, this would appear to be unlikely in this case, since the minor transition was detectable with McAbs which recognize epitopes dependent on either the tertiary or the quaternary structure of the polymer (discussed later). Thus the pre-denaturational intermediate appears to be the result of a global interaction between the fimbriae and GdnHCl, perhaps due to the GdnHCl penetrating the protein interior and attenuating intramolecular forces, as proposed for liver alcohol dehydrogenase (Strambini & Gonnelli, 1986).

(b) **Dissociation of the fimbriae.** At concentrations of GdnHCl at > 2.0 M (pH 10.5) there was a decline in the intensity of light scattered by Fim2 + 3, indicating a decrease in fimbrial size (Fig. 4). Above 3.2 M-GdnHCl, only fimbrial subunits were detectable by gel filtration with Superose 6, and polymers could not be observed by electron microscopy (results not shown); these findings indicate the loss of fimbrial quaternary structure to yield monomeric subunits. A comparison of Figs. 3(a) and 4 reveals that dissociation measured by light scattering commences at a GdnHCl concentration below that required for unfolding detectable by either fluorescence or c.d., which implies that dissociation of the fimbriae precedes denaturation. Similar distinct dissociations of quaternary structure prior to unfolding have previously been found for pig heart fumarase (Kelly & Price, 1991) and for the dimer → monomer transition of rabbit muscle pyruvate kinase (Doster & Hess, 1981). A separate step for dissociation in the unfolding pathway of the fimbriae is also consistent with the likely pathway of assembly in vivo. In this context, E. coli K99 fimbriae, which have primary structure similarities to B. pertussis fimbriae (Livey et al., 1987), including a tyrosine residue essential for K99 biosynthesis (Simons et al., 1990), are assembled in the periplasm from folded subunits which possess a native-like conformation (Bakker et al., 1991).

(c) **Subunit denaturation at pH 10.5.** Unfolding of Fim2 + 3 subunits measured by c.d. (225 nm) or fluorescence proceeds as a single-step major transition in the GdnHCl concentration range 2.8–3.6 M (Fig. 3a). The c.d. spectrum of Fim2 + 3 obtained in 3.6 M-GdnHCl (Fig. 5) shows the loss of all Cotton effects associated with the native fimbriae. The small positive peak found near 250 nm in the c.d. spectrum of denatured Fim2 + 3 is probably due to tyrosinate, although the presence of deprotonated tyrosine was not detectable by fluorescence.

**Antibody recognition of Fim2 + 3 denatured by GdnHCl at pH 10.5**

The effects of different GdnHCl concentrations on the reactivity of Fim2 + 3 with several McAbs was measured by c.l.i.s.a. The transitions obtained with antibodies 51/24, Agg3A and Agg3B (Fig. 6) were closely correlated with the subunit unfolding transition derived by both fluorescence and c.d. (Fig. 3a). This suggests that the corresponding fimbrial epitopes for these antibodies are primarily determined by the tertiary structure of the subunit. In contrast, antibodies 51/21 (Fig. 6) and Agg2A (results not shown) indicated transitions at GdnHCl concentrations which were intermediate between those required for light scattering (Fig. 4) and fluorescence or c.d. transitions (Fig. 3a). This implies that the epitope(s) for McAbs 51/21 and Agg2A are either assembled from the quaternary structure of the fimbriae or located in a protein domain which unfolds at a slightly lower GdnHCl concentration than the remainder of the subunit. However, with regard to the latter, there is no indication from the transitions derived here either by the spectroscopic methods or by the reactivity with the remaining antibodies (Fig. 6) to suggest that subunit unfolding involves more than one step. Therefore it is likely that McAbs 51/21 and Agg2A recognize an epitope(s) which is assembled from more than one fimbrial subunit. Epitopes primarily determined by the quaternary structure of fimbriae have previously been identified for both E. coli
Table 1. E.l.i.s.a. titres for antibody recognition of Fim2 + 3 preparations

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Native Fim2 + 3</th>
<th>3.2 M-GdnHCl Fim2 + 3</th>
<th>Reassociated Fim2 + 3</th>
<th>5 M-GdnHCl Fim2 + 3</th>
<th>Refolded Fim2 + 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>51/21</td>
<td>&gt; 300000</td>
<td>&lt; 18</td>
<td>&lt; 18</td>
<td>&lt; 18</td>
<td>&lt; 18</td>
</tr>
<tr>
<td>51/24</td>
<td>&gt; 100000</td>
<td>&gt; 100000</td>
<td>&gt; 300000</td>
<td>&lt; 18</td>
<td>&lt; 18</td>
</tr>
<tr>
<td>Agg3A</td>
<td>1079</td>
<td>NT</td>
<td>NT</td>
<td>&lt; 18</td>
<td>&lt; 18</td>
</tr>
<tr>
<td>Agg3B</td>
<td>&gt; 500000</td>
<td>38389</td>
<td>50316</td>
<td>&lt; 18</td>
<td>&lt; 18</td>
</tr>
<tr>
<td>Anti-native</td>
<td>&gt; 100000</td>
<td>11511</td>
<td>63652</td>
<td>&lt; 18</td>
<td>&lt; 18</td>
</tr>
<tr>
<td>Anti-reassociated</td>
<td>&gt; 900000</td>
<td>&gt; 400000</td>
<td>&gt; 400000</td>
<td>91618</td>
<td>NT</td>
</tr>
<tr>
<td>Anti-18A</td>
<td>&lt; 36</td>
<td>2955</td>
<td>1583</td>
<td>1867</td>
<td>690</td>
</tr>
</tbody>
</table>

Fig. 6. Antibody recognition of Fim2 + 3 denatured in GdnHCl, pH 10.5

Fimbriae were incubated in the indicated GdnHCl concentration, and aliquots of 2 μg of protein/ml were immobilized on to e.l.i.s.a. plates. Recognition by McAbs 51/21 (●), 51/24 (△), Agg3A (□) and Agg3B (▼) was measured as described in the Experimental section.

987p (Schifferli et al., 1987) and type 1 pili (Abraham et al., 1983), and have previously been proposed for B. pertussis fimbriae (Li et al., 1988).

Renaturation and reassociation of fimbrial subunits

Attempts at renaturing and reassociating denatured subunits into fimbrial polymers which were visible in the electron microscope and recognized by most McAbs were unsuccessful. The final product of most refolding regimes studied (e.g. step dialysis or dilution, under various conditions of temperature, pH and GdnHCl, urea and protein concentrations) showed decreased recognition by the anti-peptide antibody (Table 1), which indicates that some refolding had occurred. However, of the anti-native protein McAbs, only antibody 51/24 was able to bind weakly to the refolded product (Table 1). At high protein concentrations, similar to those used for the refolding of recombiant Fim2 (Walker et al., 1990), non-specific aggregates were produced by Fim2 + 3.

The Fim2 + 3 dissociation was found to be partially reversible by desalting with 0.05 M-phosphate/0.1 M-NaCl buffer, pH 7.2, provided that denaturation in > 3.2 M-GdnHCl had not occurred. The reassociated protein consisted of polymers which were visible in the electron microscope (results not shown), but which appeared shorter than native fimbriae. When injected into mice the reassociated protein induced polyclonal antibodies which reacted with a high titre against native Fim2 + 3 (Table 1). The reassociated protein bound McAbs 51/24 (specific for Fim2) and Agg3B (specific for Fim3) with a titre marginally greater than that obtained with 3.2 M-GdnHCl-dissociated Fim2 + 3 as the antigen (Table 1). However, McAb 51/21, which may be dependent on quaternary structure, did not recognize the re-associated fimbriae (Table 1), indicating that only a partially native structure was obtained from reassociation of the subunits. This contrasts with studies of E. coli type 1 pili, where, with the addition of Mg2+ during dialysis, GdnHCl-dissociated subunits can be reassociated to recover quaternary-structure-dependent epitopes (Abraham et al., 1983).

Denaturation of separate Fim2 and Fim3

All three fimbrial preparations, Fim2, Fim3 and Fim2 + 3, possess similar pre-denaturational and dissociation steps in the GdnHCl denaturation pathway (Figs. 4 and 7). However, for Fim2 and Fim3, subunit unfolding commenced at 2.2 M-GdnHCl (Fig. 7), which is in contrast to Fim2 + 3 where unfolding

Fig. 7. Comparison of the fluorescence-derived denaturation transitions of Fim2, Fim3 and Fim2 + 3

The tyrosine fluorescence of 20 μg of protein/ml was measured for Fim2 (●), Fim3 (▼) and Fim2 + 3 (△) following incubation in GdnHCl (pH 10.5).
commenced at 2.8 mM-GdnHCl. This difference in subunit stability to GdnHCl was also demonstrated by McAb binding to the respective proteins exposed to a range of GdnHCl concentrations (results not shown). The enhanced stability of the subunits in the Fim2+3 preparation was not due to the method of purification, since Fim2+3 prepared by either homogenization or urea extraction of whole cells (the procedure used for separate Fim2 and Fim3) retained the enhanced stability to GdnHCl (results not shown). It is difficult to explain this apparent difference in conformational stability. Perhaps the fimbiae in *B. pertussis* strains expressing Fim2+3 follow a modified folding pathway in vivo compared with the fimbiae of strains expressing a single phenotype.

**Conclusion**

GdnHCl-induced denaturation of *B. pertussis* fimbiae consists of three steps, which are discernible by either spectroscopic methods or recognition with antibodies. Below 1.0 mM-GdnHCl the fimbiae exhibit a pre-denaturational change in conformation, which is followed by dissociation of the polymer into subunits over the 2.0-3.2 mM-GdnHCl range. Unfolding of the resultant monomer appears to be co-operative, since c.d., fluorescence and antibody recognition (using tertiary-structure-dependent McAbs) indicated similar denaturation transitions. The only observable reversible step along the denaturation pathway was the partial reassociation of folded subunits, although it is likely that the pre-denaturational change is also reversible (Creighton, 1978). The limited reversibility of the dissociation may reflect a requirement *in vivo* for a chaperone-like protein involved in polymer assembly. A periplasmic protein with this function has recently been shown to be necessary for the formation of *E. coli* K88 and K99 fimbiae from their folded subunits (Bakker et al., 1991). The absence of any biologically active polymer resulting from the attempted refolding and reassociation of denatured subunits is consistent with the attempted renaturation of other oligomeric proteins, such as mitochondrial aspartate aminotransferase (West & Price, 1990) and pig heart citrate synthase (West et al., 1990) from their denatured subunits. Furthermore, it suggests that *in vivo* a chaperone-like protein may also be involved in the folding of the fimbrial subunit.

We are grateful both to Professor Tony Atkinson for his reading of the manuscript and many helpful discussions, and to Dr. Ted Ashworth for his assistance with the immunological procedures.

**REFERENCES**


Received 19 September 1991/18 November 1991; accepted 2 December 1991

1992