Structure and Stability of Protein H and the M1 Protein from *Streptococcus pyogenes*. Implications for Other Surface Proteins of Gram-Positive Bacteria†

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ABSTRACT: M proteins and other members of the M protein family, expressed on the surface of *Streptococcus pyogenes*, bind host proteins such as immunoglobulins, albumin, and fibrinogen. Protein H and the M1 protein are expressed by adjacent genes and both belong to the M protein family. In this work, the structure and stability of these two proteins have been investigated. As judged from sequence analysis and circular dichroism spectroscopy, the proteins are almost entirely in an α-helix conformation. The amino acids are arranged in a seven-residue (heptad) repeat pattern along the greater part of the proteins. These observations support the previously accepted model of M proteins as coiled-coil dimers. However, it was also found that the structures of both proteins were thermally unstable; i.e., the content of helix conformation was greatly reduced at 37 °C as compared to 25 °C or below. Together with previous findings that these proteins appear as monomers at 37 °C and dimers at low temperatures, the results suggest that the coiled-coil dimers are unfolded at 37 °C. The heptad patterns of protein H and the M1 protein showed a nonoptimal distribution of residues expected for a coiled-coil conformation. This is a possible explanation for the low thermal stability of the proteins. It was also demonstrated that the proteins were stabilized in the presence of the ligands IgG and/or albumin. Protein H and M1 protein show a high degree of sequence similarity in their C-terminal regions, and a fragment from this region displayed a high content of helix conformation, whereas fragments from the nonsimilar N-terminal parts did not adopt any stable folded structure. Thus, the C-terminal parts, which are conserved within the M protein family, may constitute a framework for the formation of the parallel helical coiled-coil structure, and we propose that the less stable N-terminal part may also participate in antiparallel interaction with M proteins on adjacent bacteria. The results suggest that temperature fluctuations in the environment could change the properties of bacterial surface proteins, thereby affecting the molecular interactions between the bacterium and its host.

The M protein family is a large group of fibrous streptococcal surface molecules that share similar C-terminal halves proximal to the cell surface and have highly variable N-terminal halves distal to the cell surface. M proteins constitute one of the key virulence factors of *Streptococcus pyogenes* due to their antiphagocytic properties and their capacity to induce host cross-reactive antibodies [for references see Fischetti (1989) and Kehoe (1994)]. Ig-binding[1] proteins from *S. pyogenes* were originally regarded as a separate group of molecules, but sequence analysis showed that these proteins are structurally closely related to M proteins (Heath & Cleary, 1989; Frithz et al., 1989), and they are now regarded as members of the M protein family. In some cases, M proteins have also been shown to bind Ig (Schmidt & Wadström, 1990; Retnoningrum et al., 1993; Retnoningrum & Cleary, 1994; Åkesson et al., 1994). The role for Ig binding in virulence and pathogenesis is not yet understood, but most clinical isolates of *S. pyogenes* do express these surface molecules (Lindahl & Stenberg, 1990).

Sequence analysis of M proteins has revealed similarities to tropomyosin, myosin, laminin, and/or keratin, with a characteristic seven-residue (heptad) repeat structure (Hosein et al., 1979; Fischetti, 1989). Furthermore, antibodies against M proteins can cross-react with these proteins (Kaplan et al., 1962; Dell et al., 1991; Vashishtha & Fischetti, 1993). Electron microscopy and ultracentrifugation studies reveals that M6 protein is a dimeric 50−60-nm-long fibrillar molecule, which contains approximately 70% α-helix as estimated from circular dichroism spectra (Phillips et al., 1981). In analogy with the mammalian fibrous proteins, a two-chain coiled-coil structure has therefore been proposed for the M proteins (Phillips et al., 1981).

Coiled-coil proteins have a characteristic seven-residue (heptad) repeat, $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, where positions *a* and *d* are normally occupied by hydrophobic residues (Hodges et al., 1972; Cohen & Parry, 1990) and positions *e* and *g* by oppositely charged residues (McLachlan & Stewart, 1975; Stone et al., 1975). Supercoiled α-helices have approxi-

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[1] Abbreviations: Ig, immunoglobulin; Fc, constant region of the heavy Ig chain; CD, circular dichroism; HSA, human serum albumin; T_m, temperature of thermal melting; UV, ultraviolet.

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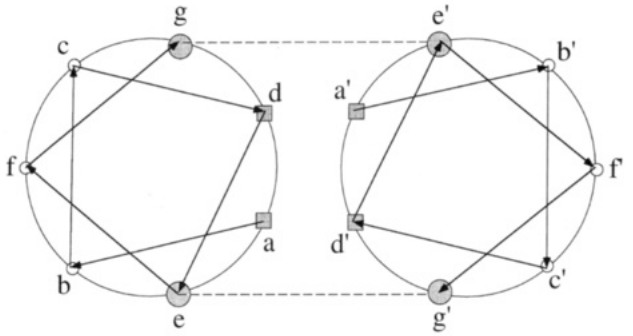

FIGURE 1: Helical wheel projection of an α-helical coiled-coil structure as viewed from the N-terminus. The amino acids in the heptad are labeled a−g. The two wheels represent the two chains of the coiled-coil dimer. Positions a and d interact with positions a′ and d′ on the adjacent chain. Interhelical ion pairs can be formed between positions e and g′ and between e′ and g and are represented with dashed lines.

mately 3.5 residues/turn, which places the hydrophobic residues on one side of the helix (Figure 1) (Crick, 1953). Interactions between residues in these positions provide the major force in the formation of a two-chain coiled-coil structure. Ion pairs between the *e* and *g* positions in the interacting chains are, however, also important for the stability as well as the orientation of the coiled-coil conformation (Zhou et al., 1994; Monera et al., 1994).

Protein H (Åkesson et al., 1990) and the M1 protein (Åkesson et al., 1994) are two IgG-binding proteins of the M protein family that are coexpressed on the surface of the AP1 strain of *S. pyogenes*. Besides its affinity for IgG, protein H also has affinity for albumin and FNIII domains (Åkesson et al., 1994; Frick et al., 1994, 1995), whereas the M1 protein also binds albumin and fibrinogen. The IgG, albumin, FNIII, and fibrinogen binding sites have been localized to different regions of the two bacterial molecules. It has recently been shown that the binding of IgG to protein H is temperature-dependent, with high affinity for IgG at 4 and 22 °C, but surprisingly, no affinity at physiological temperature (37 °C) (Åkerström et al., 1992). It was also shown that protein H exists as a dimer at these lower temperatures, while at 37 °C it occurs as monomers, indicating that dimerization of protein H is a prerequisite for the binding of IgG. Similar behavior was also observed for another Ig-binding protein, protein Arp4, and more recently also for protein Sir and the M1 protein (Cedervall et al., 1995).

In this report we have further characterized the structure and stability of protein H and the M1 protein. The results obtained are discussed and related to the structural and functional properties of other cell surface proteins of Gram-positive bacteria.

MATERIALS AND METHODS

Proteins. Protein H was prepared by expression of the corresponding gene in *Escherichia coli* as previously described (Åkesson et al., 1990; Gomi et al., 1990). The A fragment of protein H was expressed (Frick et al., 1994) and purified using size-exclusion chromatography (Frick and Wikström, unpublished work). The M1 protein and the M1 protein fragments were prepared as described by Åkesson et al. (1994). Polyclonal human IgG and human serum albumin were purchased from Sigma Chemical Co. These proteins were further purified using ion-exchange chromatography. The parvalbumin sample was a generous gift from Eva Thulin, Lund University.

Physicochemical Characterization. The Stokes radius of protein H was measured using gel chromatography as described by Laurent and Killander (1964) at 20 and 37 °C. Bovine serum albumin (BSA) was used as a standard with known Stokes radius. The M_r of the denatured protein H was measured by gel chromatography in 6 M guanidine hydrochloride using BSA and ovalbumin as M_r standards. Frictional ratios were calculated as described previously for protein L (Åkerström & Björck, 1989).

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were recorded on a Jasco J-720 spectropolarimeter equipped with a thermostated cell holder. The spectra were recorded in the far ultraviolet (UV) region (260−190 nm) in cells with path lengths of 0.1 and 1.0 cm. The experiments were recorded in PBS buffer (8 mM sodium phosphate, 1.5 mM potassium phosphate, 0.12 M sodium chloride, and 2.7 mM potassium chloride), pH 7.4. The concentration of the protein samples was determined by quantitative amino acid analysis. Spectra were acquired at a scan speed of 10 nm/min. and a 4-s response time. The solvent dichroic absorbance was subtracted using the Jasco software. The thermal unfolding curves were run both for the whole spectrum region and at a single wavelength (222 nm) characteristic for α-helical structures. The temperature was increased from 4 to 90 °C at a scan rate of 50 °C/h. A slower scan rate (20 °C/h) was also tested but was shown to give the equivalent unfolding behavior. The higher scan rate was therefore used for all experiments.

Secondary Structure Estimation from CD Spectra. The program SELCON (Sreerama & Woody, 1993) was kindly made available by the authors. This program estimates the amount of secondary structure using a database of 17 proteins with well-characterized three-dimensional structures.

Equilibrium Urea Denaturation. The equilibrium denaturation experiments on protein H and the M1 protein in urea were performed in PBS buffer, pH 7.4, from 0 to 5 M urea at a protein concentration of 4.2 μM. The samples were monitored at 2 °C to prevent any effect of thermal denaturation of the sample. The unfolding was followed at a single wavelength (222 nm). The values for $[\text{urea}]_{50\%}$, the concentration of urea at which 50% of the protein is unfolded, and the free energy of unfolding were calculated from (Clarke & Fersht, 1993)

$$\Delta G_{\text{U-F}}^{\text{D}} = \Delta G_{\text{U-F}}^{\text{H}_2\text{O}} - m[\text{urea}] \quad (1)$$

$$K_{\text{U-F}} = (F_{\text{F}} - F)/(F - F_{\text{U}}) \quad (2)$$

where m is the slope of the plot, F_{F} is the intensity of the CD of the folded state, F_{U} is the intensity of the CD of the unfolded form, and F is the intensity of the CD at a given urea concentration.

Computational Sequence Analysis. The secondary structure analysis of protein H and the M1 protein was performed with the method of Rost et al. (1993, 1994, 1995). The sequence Fourier transform and probabilities were determined with the method of McLachlan and Stewart (1976). An algorithm described by Parry (1982) was used to predict coiled-coil propensities based on the statistical preference of different amino acids for each position of the heptad repeat. The sequences were analyzed using a computer

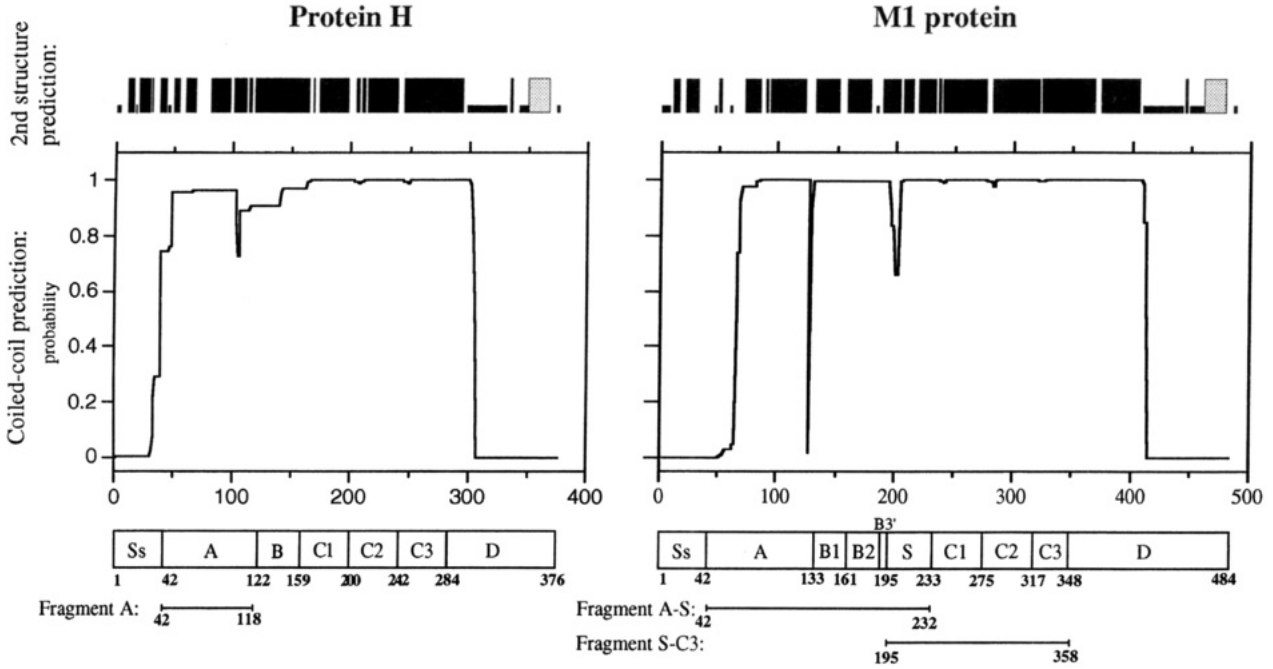

FIGURE 2: Schematic representations of the primary structure of protein H and the M1 protein. Peptide fragments of the two proteins used in this work are depicted (lower). Prediction of secondary structure content are shown at the top with α-helix shown as solid high bars, loop structures as solid low bars, and transmembrane regions as shaded bars. The probability of coiled-coil formation is shown in the middle of the figure.

program ("coiledcoil"; Lupas et al., 1991) based on this algorithm.

RESULTS

Secondary Structure Analysis. Secondary structure analysis of protein H indicated that the extracellular part (sS, A, B, and C regions) and part of the D region comprising amino acids 1-296 is predominantly (64%) α-helical (Figure 2). The proline-rich sequence further toward the C-terminus (amino acids 299−350), a region likely to be located within the peptidoglycan of the cell wall, has a high content of nonhelix structure. This region ends with the conserved bacterial cell wall LPSTGE motif and is followed by a helical hydrophobic region (amino acids 351-370) typical of a transmembrane sequence (Rost et al., 1995). The analysis of the M1 protein reveals a similar secondary structure profile, with the extracellular part of the molecule (amino acids 1−404) consisting of 76% helical structure (Figure 2).

Prediction of Coiled-Coil Structure. The sequence of protein H (Gomi et al., 1990) was analyzed by the statistical method described by Parry (1982). As depicted in Figure 2, a high probability for α-helical coiled-coil structure was predicted for almost the entire extracellular part (amino acids 49−302). The probability curve indicates that the A and B regions of protein H do not fit a coiled-coil structure as well as the C repeats. For the M1 protein, the major extracellular part of the molecule is also predicted to adopt a coiled-coil structure (amino acids 63−405). The signal peptide, and also a short part of the N-terminus of the mature protein, have zero coiled-coil probability. Several other sequences from bacterial cell wall proteins were analyzed for their contents of α-helical coiled-coil structure. Some are shown in Figure 3. The result of the analysis show that the extracellular parts of the various members of the M protein family show a high probability for coiled-coil structure, whereas the cell wall- and transmembrane-spanning regions do not. Thus, besides the M proteins, the prediction analysis suggests that several of the M protein-related Ig-binding proteins, FcRA76 (Heath & Cleary, 1989) (amino acids 90−358), protein Mrp (O'Toole et al., 1992) (amino acids 55−425), protein Arp (Frithz et al., 1989; Lindahl & Åkerström, 1989) (amino acids 57−330), and protein Sir (Stenberg et al., 1994) (amino acids 39−308), as well as the plasminogen-binding protein PAM (Berge & Sjöbring, 1993) (amino acids 50−334) and MLC36 (Ben Nasr et al., 1994) (amino acids 53−425) adopt a coiled-coil structure. The length of the non-coiled-coil sequence of the N-terminal part of the mature protein seems to vary between different M proteins, from zero amino acids for protein H to approximately 50 residues for FcRA76. The M protein family can be divided into two major classes depending on the type of repeats found N-terminally of the wall spanning region. These two types of repeats are designated A and C (O'Toole et al., 1992), and are shown in Figure 3, panels A and B, respectively. As shown, the conserved C-terminal part of the extracellular region for both classes of the M protein family fit well in a coiled-coil structure. No general pattern can be seen for the less conserved N-terminal part of these molecules. M24 protein and protein PAM are, for example, given very high probability scores for the whole region, whereas for M5, M6, and M12 only segments receive the highest probability score, indicating that the heptad structures of the other regions are less optimal. This may be related to the fact that these M proteins have insertions (or deletions) resulting in distortions of their heptad structures (Khandke et al., 1987, 1988; Fischetti et al., 1988; Manjula et al., 1991).

Proteins A, G, and L are Ig-binding bacterial proteins which do not belong to the M protein family. The three-dimensional structures of the immunoglobulin-binding repeats of proteins A, G and L have been determined; they are found to be folded into well-defined globular structures (Gouda et al., 1992; Gronenborn et al., 1991; Lian et al., 1992; Wikström et al., 1993, 1994). As expected, no coiled-coil structure is predicted for these parts of the proteins, but

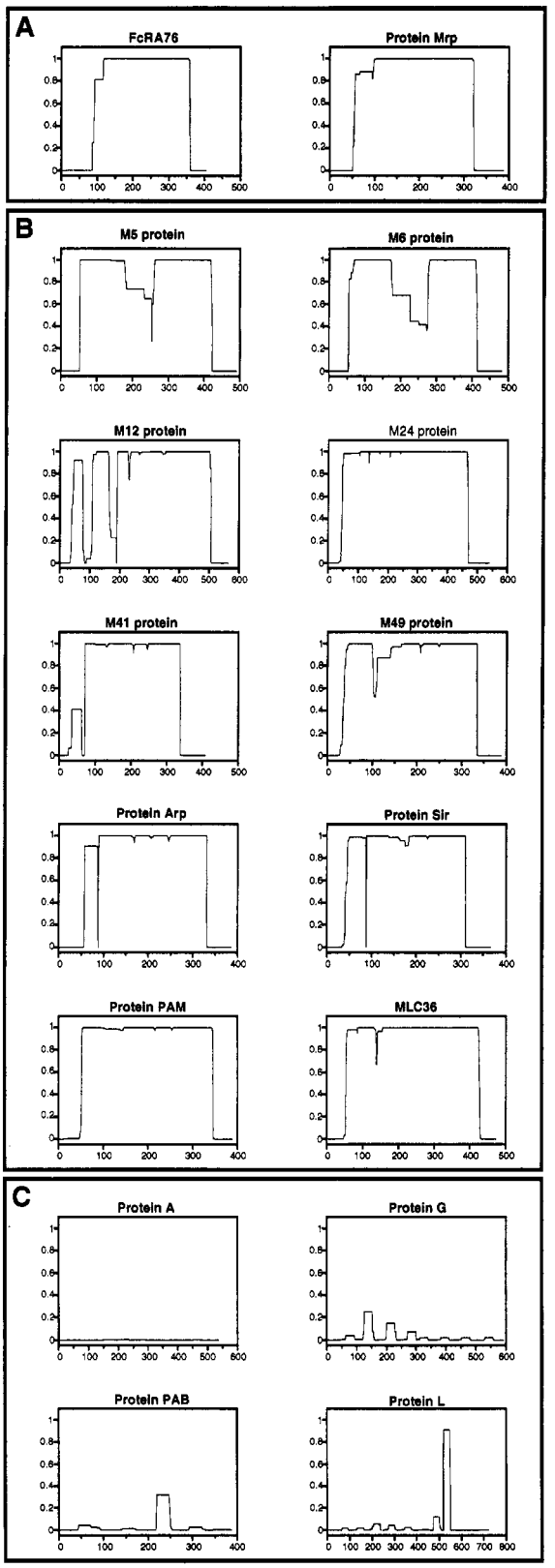

FIGURE 3: Probability for the formation of coiled-coil structure for a selected set of bacterial surface proteins. (A) Proteins with A repeats: FcRA76 (Heath & Cleary, 1987) and Mrp (O'Toole et al., 1992). (B) Proteins with C repeats: M5 (Miller et al., 1988), M6 (Hollingshead et al., 1986), M12 (Robbin et al., 1987), M24 (Mouw et al., 1988), M41 (Podbielski, 1993), M49 (Khandke et al., 1988), proteins Arp (Frithz et al., 1989; Lindahl & Åkerström, 1989), Sir (Stenberg et al., 1994), and PAM (Berge & Sjöbring, 1993), and MLC36 (Ben Nasr et al., 1994). (C) Bacterial surface proteins not considered as members of the M protein family: proteins A (Uhlén et al., 1984), G (Guss et al., 1986), PAB (de Chateau & Björck, 1994), and L (Kastern et al., 1992).

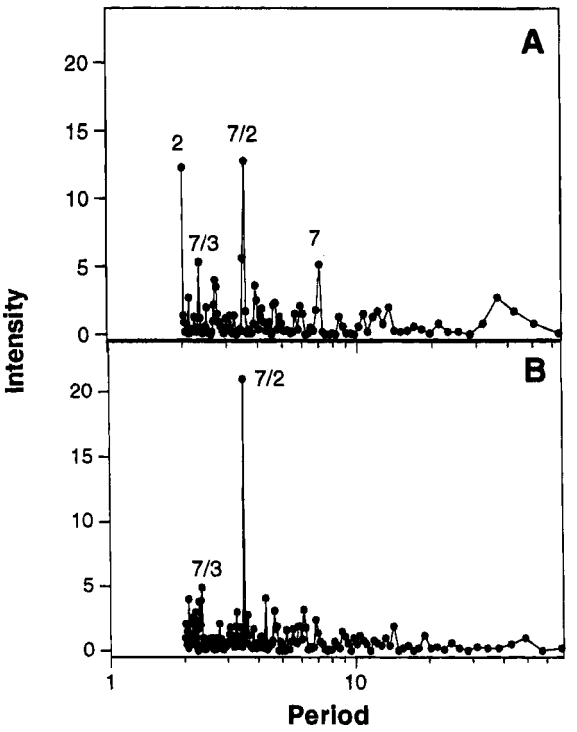

FIGURE 4: Fourier analysis of the periodicity for the hydrophobic residues L, V, Y, and I in protein H (A) and M1 protein (B). Period values are presented in a logarithmic scale with significant peaks (i.e., a probability of random occurrence that is less than 0.01) labeled by the period values.

the C2 repeat (amino acids 520–548) of protein L have a coiled-coil probability >0.8 (Figure 3C). The analysis also included the albumin–binding protein PAB (de Chateau & Björck, 1994), the IgD-binding protein D (Janson et al., 1991), and the fibronectin-binding protein F (Hanski & Caparon, 1992). None of these bacterial surface proteins, which are not structurally related to the M protein family, have significant probabilities for coiled-coil structure (not shown).

Heptad Structure of Protein H and the M1 Protein. The amino acid residues in the region 40-303 of protein H and the region 63-405 of the M1 protein were analyzed for heptad sequence periodicities by Fourier analysis (MacLachlan & Stewart, 1976). For the hydrophobic residues (L, I, V, and Y) of protein H, significant intensities were detected for the periodicity of seven and for harmonics of heptad repeats: 3.51 and 3.48 (~7/2), and 2.33 (~7/3) and the periodicity of 2 (Figure 4A). The hydrophobic amino acids of the M1 protein also showed significant intensities for heptad harmonics, especially at 3.5 (~7/2) (Figure 4B). Further examination of the amino acid sequences of protein H and the M1 protein revealed patterns specific to α-helical coiled-coil proteins. These are characterized by the presence of the form $(a\cdot b\cdot c\cdot d\cdot e\cdot f\cdot g)_n$ (McLachlan & Stewart, 1975) with the *a* and *d* positions being preferentially occupied by apolar amino acids (Figure 1). The periodicity, which extends from amino acid 42 through 297 of protein H (Figure 5A), is disrupted in two positions, after amino acids 103 and 139. For the M1 protein the periodicity starts at position 63 and extends to amino acid 405 and is disrupted in three locations, after amino acids 133, 196, and 392 (Figure 5B).

For protein H and the M1 protein, respectively, approximately 62% and 50% of the residues found in the *d* position are apolar (predominantly leucine), whereas only

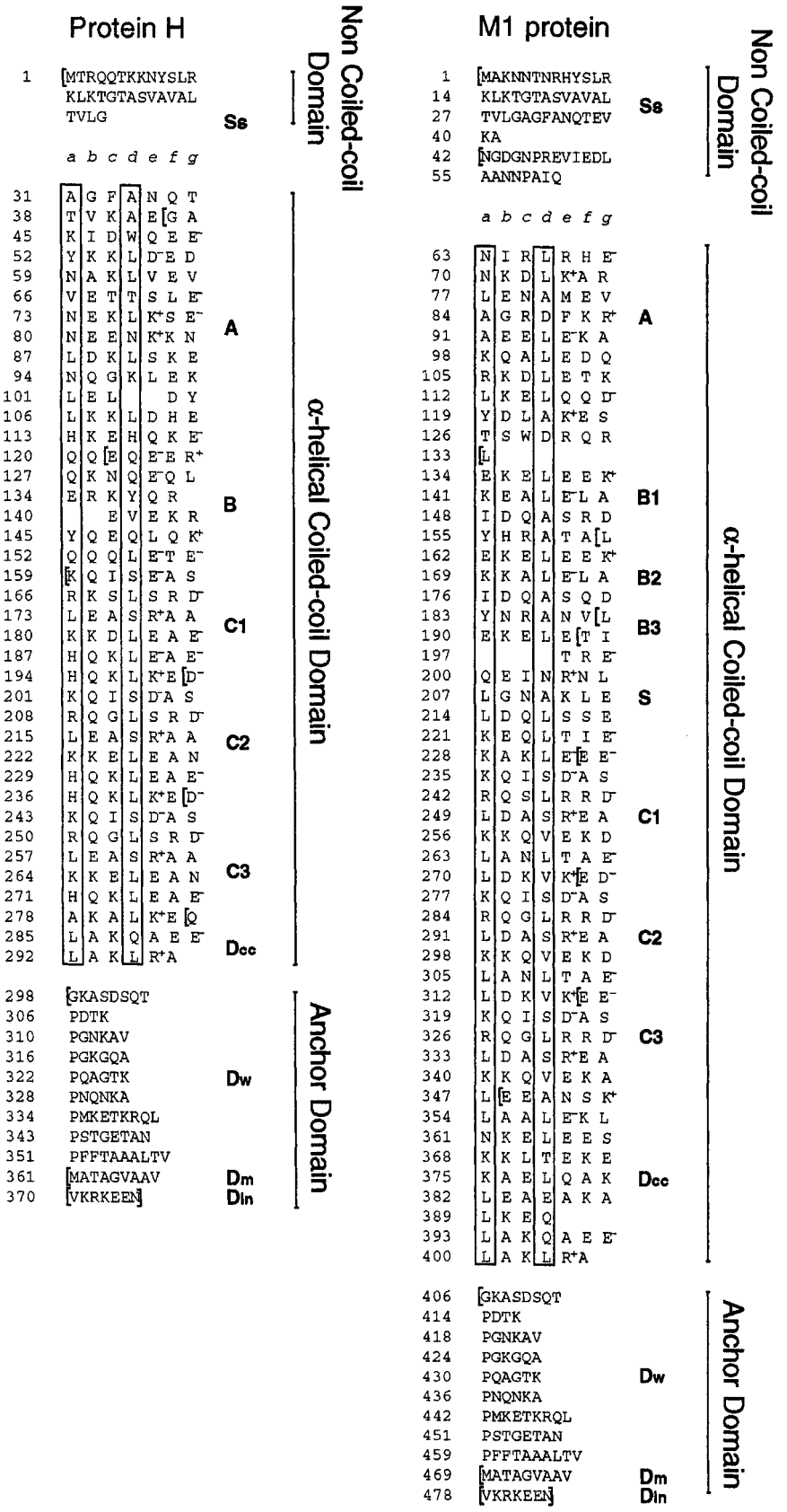

FIGURE 5: Heptad structures of protein H (left) and the M1 protein (right). The domains are named as in Figure 2 except for the D domain, which is divided into D_{cc} (coiled-coil), D_w (wall spanning), D_m (membrane-associated), and D_{in} (intracellular) regions. Brackets in the sequences indicate the beginning of each domain. The sign of the charged residues involved in potential electrostatic interactions between the e and g positions are denoted.

34% and 35% apolar residues are found in the *a* position. In this position a high distribution of basic residues is seen (26% and 35%, respectively), which is a common property for all described M proteins that differentiates them from other described α-fibrous proteins (Conway & Parry, 1990). When the distribution of amino acids in the A, B, and C domains of protein H and the M1 protein is analyzed, similarities with other M proteins are evident. Thus, the A domain of protein

H has a high content of asparagine (40%) in the *a* position and of apolar residues (50%) in the *d* position. A similar pattern has previously been found in the corresponding domains (subdomain I) of proteins M5, M6, and M24 (Manjula et al., 1991). The high occurrence of glutamine found in the *a* and *d* position of the B domain of protein H can be seen in the subdomain II of M49 as well. The B segment is also a highly charged region which results in a lower probability for the formation of a coiled coil in this part of protein H (Figure 2). Figure 5A also shows that the B portion does not contain the expected leucine residues in either the *a* or the *d* position. Interestingly, when the sequence representing the B segment was aligned with other sequences in the database, a significant identity score (41%) was found for the protein trichohyalin (Fietz et al., 1993). Biophysical studies of this protein indicate that it exists as a single extended α-helical structure in solution (Lee et al., 1993). The A domain of the M1 protein has a high content of apolar residues in *a* (50%) and *d* (80%) positions which has previously been observed in M12, M49, and M57, and the high frequency of apolar residues in *a* and *d* position in the BS region has earlier been reported for the M12 and M57 proteins (Manjula et al., 1991). Finally, the C repeats of proteins H and M1 show have a high sequence similarity with C repeats of other M proteins of the C class (Manjula et al., 1991). This part of the molecule has a high proportion of basic residues (50%) in the *a* position in both protein H and the M1 protein, whereas a marked prevalence of histidine (27%) is found in this position only in protein H. Both proteins have a high portion of apolar residues (67% and 60%, respectively) and serine (33% and 40%, respectively) in the *d* position.

In 34 of the heptads identified in protein H, interhelical ionic interactions (Figure 1) are likely to occur between residues in the *g* and *e* positions of one chain with the *e'* and *g'* positions of the other chain. Twenty-four of them could result in electrostatic attractions, while 12 have the potential to induce electrostatic repulsions (Figure 5A). Similar results are found for the heptads of the M1 protein, which contain 26 favorable ion pairs and eight unfavorable (Figure 5B). Analysis of the described heptad structures of M6, M12, M24, and M57 (Fischetti et al., 1988; Manjula et al., 1991) showed similar ratios between repulsive and attractive interactions in the *e* and *g* positions, 2/20, 6/28, 12/42, and 12/18, respectively.

Physicochemical Properties of Protein H. The Stokes radius of protein H was determined from gel filtration experiments at 20 and 37 °C. From these values and the molecular mass from the amino acid sequence, the frictional ratio was calculated at the two temperatures (Table 1). The frictional ratio was calculated with the assumption that at the lower temperature protein H is in a predominantly dimeric state, whereas at the higher temperature the molecule is found in a monomeric state. The frictional ratio is in both cases indicative of a highly elongated structure (Cantor & Schimmel, 1980). Interestingly, the frictional ratio representing the dimeric form is higher than the monomer form, suggesting that the dimeric coiled-coil state, observed at lower temperatures, is more elongated than the monomeric form. Since the dimeric state is expected to be represented by an elongated α-helical coiled coil, while the high-temperature conformation is considered to adopt a flexible conformation, this is not surprising.

Table 1: Physicochemical Properties of Protein H

property	value
molecular mass	
from amino acid composition[a]	38 162
from SDS-polyacrylamide gel electrophoresis	42 000
from gel chromatography in guanidine-HCl	34 700
Stokes radius	
r_S (T = 20 °C)	51.4 Å
r_S (T = 37 °C)	36.1 Å
frictional ratio	
f/f_0 (T = 20 °C)	1.84
f/f_0 (T = 37 °C)	1.63
disulfide bonds	none
absorption coefficient at 280 nm (ϵ)	3.9×10^4 cm^{-1} M^{-1} [b]

[a] The protein sequence minus signal peptide was deduced from the gene sequence (Gomi et al., 1990). [b] The M_r value of 38 162 was used.

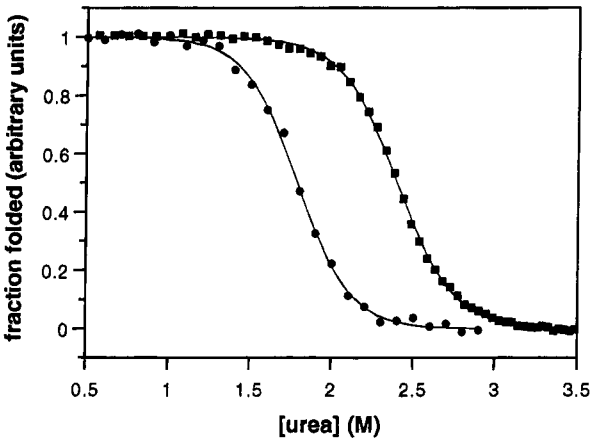

FIGURE 6: Normalized unfolding curves for protein H (●) and the M1 protein (■) as a function of the concentration of urea. Data points represent CD intensities at 222 nm and solid curves represent theoretical two-state transitions fitted to the data (based on eqs 1 and 2). The concentration of urea at which 50% of the proteins are unfolded, $[urea]_{50\%}$, was 1.79 ± 0.01 and 2.40 ± 0.01 M for protein H and the M1 protein, respectively. The ΔG_{H_2O} and m values obtained from the analysis were for protein H 25.1 ± 0.01 kJ/mol and 14.1 ± 0.40 kJ/mol^2, respectively, and for the M1 protein 31.7 ± 0.01 kJ/mol and 13.2 ± 0.14 kJ/mol^2, respectively.

Urea Denaturation. The stability of protein H and the M1 protein upon titration with urea was also measured by CD at 222 nm. Data were collected at 2 °C to prevent effects due to thermal denaturation of the sample. The data fit, in both cases, a two-state transition (Figure 6) and the entire set of data was fit to an unfolding transition curve based on eqs 1 and 2 (see Materials and Methods). The concentration midpoint for unfolding, $[urea]_{50\%}$, is 1.79 ± 0.005 M urea for protein H and 2.39 ± 0.005 M for the M1 protein, confirming the low stability of the proteins. The free energy of unfolding in water, $\Delta G_{U-F}^{H_2O}$, was 25.1 ± 0.1 kJ mol^{-1} and 31.6 ± 0.1 kJ mol^{-1} for protein H and the M1 protein, respectively.

Secondary Structure Analysis of Protein H Using CD Spectroscopy. Far-UV CD spectra of protein H were recorded at various temperatures (Figure 7A). They reveal that protein H is a predominantly α-helical protein at 4 °C, as indicated by the double minima at 207 and 222 nm, as well as the maximum at 192 nm. The α-helical content was estimated from the CD spectra to be 74% using the method described by Sreerama and Woody (1993). The ratio of molar ellipticity ($[\theta]_{222}/[\theta]_{207}$ = 1.18 is similar to that observed previously for coiled coils (Lau et al., 1984; Hodges et al., 1988, 1990; Zhou et al. 1992b,c) and different from structures with α-helices in non-coiled-coil conformations,

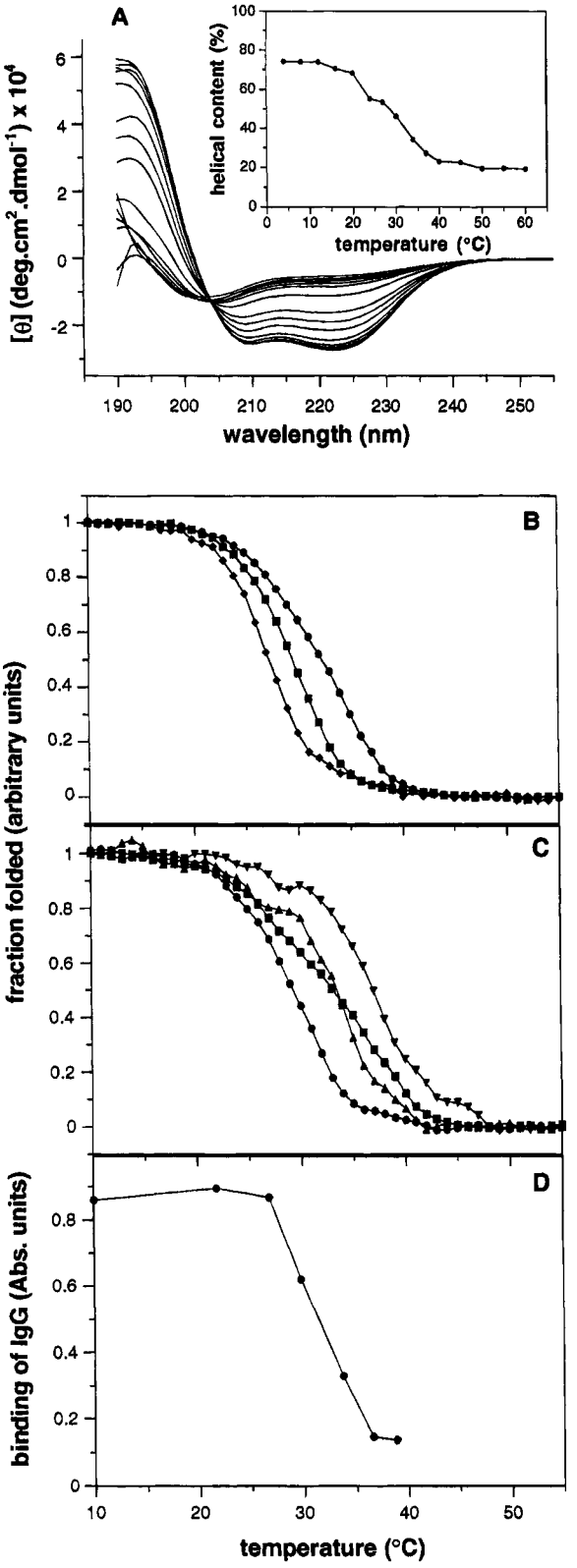

FIGURE 7: CD analysis of protein H. (A) Far-UV spectral region of the intact protein H (4.2 μM) at increasing temperatures, with the estimated helical content in the upper right corner. (B) Normalized CD signal at 222 nm for protein H at 0.42 (◆), 4.2 (■), and 65 μM (●) as a function of temperature. (C) CD at 222 nm for protein H (4.2 μM) free (●), together with IgG (▲) or HSA (■), and with both IgG and HSA (▼) as a function of temperature. (D) IgG-binding activity for protein H adapted from Åkerström et al. (1992) as a function of temperature.

in which the ellipticity at 207 nm is generally more negative than that at 222 nm (Cooper & Woody, 1990; Zhou et al., 1993). The conformation of protein H is dramatically affected by an increase in temperature. The estimated α-helical content of protein H drops from approximately 55% to 27% when the temperature is raised from 24 to 37 °C. When the thermal denaturation of protein H is followed at 222 nm, a smooth transition curve can be seen (Figure 7B), which correlates with the binding activity of IgG to protein H at various temperatures (Figure 7D). The denaturing process could be completely reversed by lowering the temperature back to 4 °C. When three different concentrations of protein H were analyzed (0.42, 4.2, and 65 μM), different melting temperatures (T_m values) could be observed, 27, 30, and 32 °C, respectively (Figure 7B). Since the melting curve was concentration-dependent, all further CD analysis of protein H were performed at a fixed concentration, 4.2 μM. CD spectra were also measured on a fragment of protein H representing the A domain (Figure 2). This domain showed a weak CD signal indicating no appreciable amount of secondary structure, and when it was heated to 90 °C no change in the ellipticity was observed (not shown). This demonstrates that the isolated A domain does not adopt a stable folded structure in solution.

Secondary Structure Analysis of the M1 Protein Using CD Spectroscopy. To further investigate its structural properties, the M1 protein was subjected to analysis by CD methods. The far-UV region was, as in the case of protein H, indicative of an α-helical structure (~70% α-helix at 4 °C) (Figure 8A). When heated, the M1 protein starts to unfold above 30 °C, and at 37 °C only 52% are in a folded state. Analogous to protein H, this correlates with the drop in affinity for IgG of the M1 protein at this temperature (Cedervall et al., 1995). The unfolding behavior was, however, different as compared to protein H, since the CD intensity continued to decrease with increasing temperature (Figure 8B). This behavior suggests that the unfolding of the intact M1 protein does not undergo a simple two-state transition. A fragment from the N-terminal portion (fragment A-S) had a weak CD signal with no indication of stable secondary structure and, as for the A domain from protein H, no change in the CD intensity was observed when the sample was heated (not shown). However, the isolated fragment of the M1 protein representing the S-C3 region was, according to the CD analysis, in an α-helical conformation with an α-helix content of approximately 85% (Figure 8C). The temperature denaturation experiment is indicative of a two-state transition with a $T_m \sim 17$ °C (Figure 8D).

Effect on the Thermal Stability of Protein H in the Presence of Ligands. The far-UV CD spectra at 4 °C were recorded for protein H alone and in the presence of equimolar concentration of different ligands: IgG, albumin, or IgG plus albumin. The concentrations were chosen such that >90% of protein H was calculated to be in complex with the ligands at low temperature. The final spectra were derived by subtracting the spectrum of the ligand(s) alone from the spectrum of the mixture of protein H and ligand(s). The final spectra at 4 °C were not changed when IgG or albumin were added, indicating that no conformational change occurs when protein H binds these ligands (data not shown). Figure 7C shows the CD thermal transition curves at 222 nm of protein H alone and with the different ligands. When IgG is added to protein H, the low-temperature conformation is stabilized, as seen by the increase of the melting temperature by 3 °C (from 30 to 33 °C). A similar effect is observed by the addition of albumin, an increase of 2 °C (from 30 to 32 °C). A synergistic effect is seen when both IgG and albumin

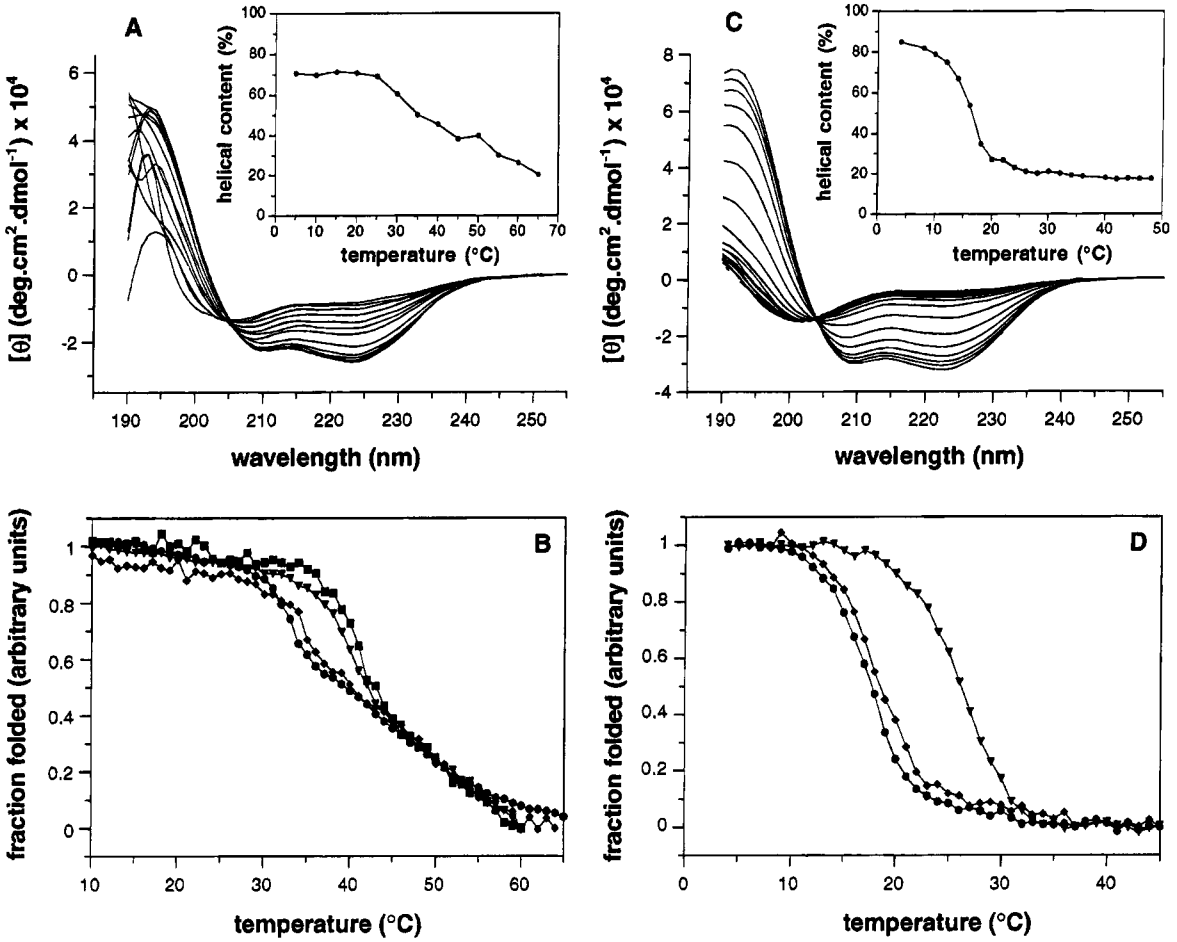

FIGURE 8: CD analysis of the M1 protein. (A) Far-UV spectral region of the intact M1 protein (4.2 μM) at increasing temperatures, with the estimated helical content in the upper right corner. (B) Unfolding curves (CD at 222 nm) for the M1 protein free (●), together with IgG (◆) or HSA (▼), and both IgG and HSA (■). (C) Far-UV spectral region of fragment S-C3 (4.2 μM) at increasing temperatures, with the estimated helical content in the upper right corner. (D) Unfolding curves (CD at 222 nm) for the S-C3 fragment free (●) and together with IgG (◆) or HSA (▼).

are added to protein H, resulting in an even higher melting temperature (37 °C), i.e., an increase of 7 °C. In a negative control experiment parvalbumin was added to the protein H sample with no effect on the melting temperature (data now shown).

Effect on the Thermal Stability of the M1 Protein and the S-C3 Fragment in the Presence of Ligands. As in the case of protein H, the thermal stability of the M1 protein was investigated without and with the ligands HSA and IgG. The change in unfolding behavior of the M1 protein upon addition of IgG and HSA is shown in Figure 8B. HSA was shown to have a much larger effect on the unfolding temperature than IgG. Also the C-terminal fragment of the M1 protein, S-C3, was investigated together with ligands (Figure 8D). The addition of HSA had a dramatic effect on the unfolding behavior of the S-C3 fragment. The transition temperature $T_m \sim 17$ °C observed for the free peptide increases to $T_m \sim 27$ °C with HSA present.

Antiparallel Alignment of the Heptad Structures of Protein H and the M1 Protein. Electron microscopy studies on streptococci reveal that M proteins of neighboring bacteria can interact with each other (Swanson et al., 1969; Philips et al., 1981; Fischetti, 1989). Our studies show that protein H and the M1 protein can fold and unfold their coiled-coil structures close to 37 °C. The low thermal stability may therefore allow these proteins to refold in an antiparallel fashion with M proteins on adjacent bacteria. Since intermolecular electrostatic interactions have been shown to be important for determining the orientation of two-stranded coiled-coils (Monera et al., 1994), we decided to examine these interactions for aligned antiparallel sequences of protein H and the M1 protein with themselves or with each other. All possible overlaps that fitted with the heptad structure of an antiparallel coiled-coil of protein H and the M1 protein were analyzed with respect to the distribution of different potential interhelical electrostatic interactions in the *g* and *e* positions. The electrostatic interactions of the antiparallel pairing of protein H with itself are in a few cases more favorable than the electrostatic interaction of the equivalent parallel alignment. The best antiparallel alignments of protein H were found to have overlaps of 120 and 141 amino acid residues, respectively (Figure 9A). This would thus involve the A and B regions, and in the latter case also the beginning of the C1 region (Figure 9C). This latter alignment results in eight attractive and two repulsive interactions. The repulsive interactions are, however, located in the N-terminus of each chain and can therefore be expected to be of less importance than the interior interactions. The same region of the parallel structure has ten attractive bonds and six repulsive interactions. The alignment of the M1 protein sequence with itself suggests that one position (an overlap of 38 residues), has potentially favorable ion interactions for the antiparallel arrangement (Figure 9B). This alignment involves the N-terminal portion of the A domain (Figure 9C).

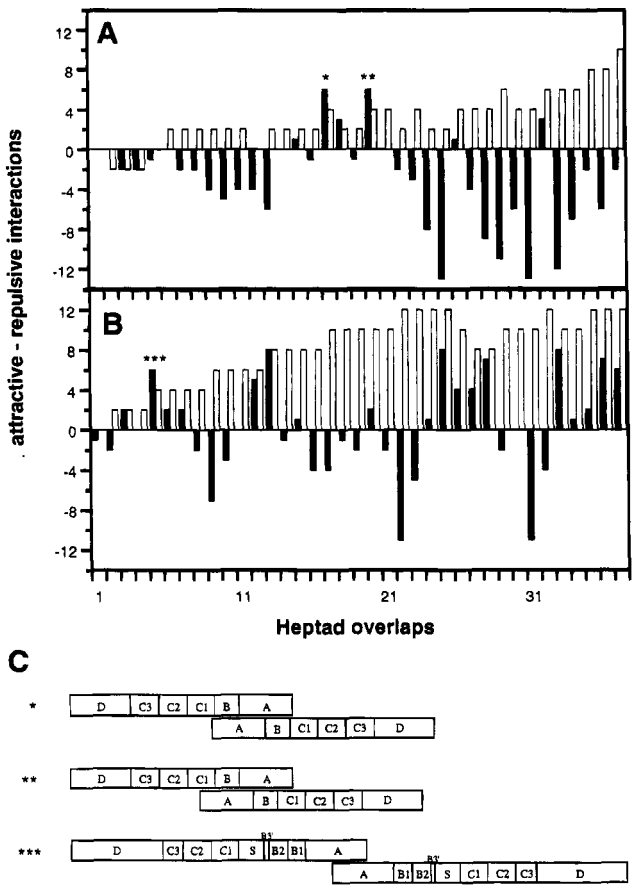

FIGURE 9: Antiparallel and parallel alignment of the heptad structure of protein H and the M1 protein. The heptad structures of protein H (A) and the M1 protein (B) were aligned in an antiparallel (filled bars) orientation (with a pairing with d′ and d pairing with a′ in the heptad structure) or in a parallel (open bars) orientation (with a pairing with a′ and d pairing with d′). An alignment of one heptad repeat overlap is interpreted as an alignment of the first N-terminal heptad repeat, a two-heptad repeat overlap is an alignment of the two first heptad repeats in the N-terminal of the protein, etc. The antiparallel and parallel alignments were analyzed for intermolecular electrostatic interactions in the e and g positions (i.e., for the antiparallel alignment g pairs with g′ and e with e′, and for the parallel alignment g pairs with e′ and e with g′) and the difference between attractive and repulsive interactions was calculated. Favorably antiparallel aligned sequences are labeled with stars and represented schematically in (C).

Finally, the alignment of protein H with the M1 protein indicates also an overlap of 68 amino acids with potentially favorable (eight attractive and no repulsive) ion interactions (not shown). These results suggest that an antiparallel coiled-coil structure could occur and may be more favorable than the parallel organization for certain regions of the proteins.

DISCUSSION

The Ig-binding proteins H, Arp, Sir, FcRA76, and Mrp as well as two plasminogen-binding proteins, proteins PAM and MLC36, were shown to share the common overall coiled-coil structure known for M proteins, and they should therefore be regarded as members of the M protein family. This prediction method distinguishes coiled-coil proteins from other α-helix-rich proteins, like protein A, and is therefore a valuable tool in the evaluation of the structural relationship between various bacterial cell wall-associated proteins.

The sequence in the IgG Fc-binding regions are different for proteins H, A, and G, although it has been shown that they all bind to the Cγ2−Cγ3 interface area of IgG (Deisenhofer, 1981; Stone et al., 1989; Frick et al., 1992; Sauer-Eriksson et al., 1995). Furthermore, the structures of these proteins also exhibit considerable differences; the protein A domains are constructed as a three-helix bundle, the protein G domains consist of a four-stranded β-sheet with a single α-helix on top, and protein H, as further evidenced in this report, have a two-stranded parallel α-helical coiled-coil structure. This indicates strongly that the three IgG Fc-binding bacterial proteins have evolved their binding capacities through convergent evolution, suggesting that these surface proteins are connected with essential microbial functions adding selective advantages to the bacteria.

Previous findings have shown that the binding of Ig to protein H, M1 protein, and proteins Arp and Sir is temperature-dependent; i.e., they bind Ig with high affinity only at temperatures <37 °C (Åkerström et al., 1992; Cedervall et al., 1995). The temperature-dependent binding was also seen at the bacterial surface. All four molecules belong to the M protein family and it is therefore possible that temperature-dependent ligand binding is a common feature of this protein family. Interestingly, our results demonstrate that the drop in binding affinity can be directly correlated to the unfolding of the coiled-coil dimer of protein H (Figure 7B,D). The temperature unfolding curve of intact protein H suggests that the protein undergoes a two-state transition from a folded dimeric coiled coil to an unfolded monomeric state at higher temperatures with a T_m of 30 °C. Such behavior is in line with what has been observed in gel-filtration experiments (Åkerström et al., 1992), where the dimeric state is observed at low temperature (4 and 10 °C), whereas monomers occur at high temperature (37 °C). As a comparison, the average T_m of unfolding, summarizing thermodynamic data of a large number of proteins (Pheil, 1986), is ~63 °C. The M1 protein was also found to be thermally unstable but had a more complex unfolding behavior. The unfolding started just above 30 °C but did not confirm to a simple two-state process (Figure 8B). This behavior of the M1 protein could be due to different unfolding temperatures in different regions of the coiled-coil structure. The regions of protein H and the M1 protein with the lowest degree of similarity are found in the N-terminal regions. The M1 protein has also an additional block of 44 amino acids in the D domain as compared to protein H. It is possible that the degree of stability in different fragments and the intact M1 protein is A-S < S-C3 < S-D < intact M1 protein. These differences could explain the different unfolding behavior of the two proteins. Furthermore, the melting temperatures were modulated by the binding of ligands to the coiled-coil structure of protein H and the M1 protein. Thus, free protein H was less stable than protein H in complex with either IgG or albumin and further stabilization was observed in the presence of both ligands, which results in an increase from <10% to ~50% (with both ligands) of folded protein H at 37 °C (Figure 7C).

As judged by CD, the N-terminal portions of protein H and the M1 protein were not able to adopt stable structures as isolated peptides. This observation supports gel chromatography experiments (Cedervall et al., 1995), which suggest that these peptides exist as monomers in solution. Interestingly, the peptide fragment containing the S domain followed by the three C repeats (S-C3) from the M1 protein was shown to be in a folded state at 4 °C (Figure 8C). The unfolding behavior of this peptide suggest a two-state transition going

from a folded helical state at low temperature to an unfolded state at higher temperatures. This fragment was also markedly stabilized by the addition of HSA, supporting previous mapping of the albumin binding to the C repeats (Frick et al., 1994; Åkesson et al., 1994). A majority of the sequenced genes of M proteins contain C repeats. This region is, in fact, together with the signal sequence and the membrane-associated part, the most well-conserved region within the M protein family. The N-terminal region, on the other hand, shows a much lower degree of sequence similarity among various M proteins. The C repeats may therefore represent a framework for the formation of the α-helical coiled-coil structure, and this could be one of the explanations why the C repeats are so highly conserved among M proteins of class C.

Electron microscopy has indicated that M proteins of adjacent bacteria may form specific end-to-end interactions involving the distal N-terminal regions (Swanson et al., 1969; Phillips et al., 1981; Fischetti, 1989). The present work shows that protein H and the M1 protein both have the ability to fold and unfold their coiled-coil structure within a small temperature range close to 37 °C. It therefore seems possible that these surface molecules could unfold and refold in an antiparallel fashion together with M proteins on adjacent bacteria. To test this hypothesis, sequences of both protein H and the M1 protein were positioned in all possible antiparallel heptad alignments. It was found that the antiparallel arrangement can indeed be more favorable than the parallel structure. Interestingly, these favorable antiparallel alignments exclusively involve residues in the N-terminal portions of the two molecules. This kind of interaction could therefore provide a mechanism for cell–cell interactions among *S. pyogenes* and may also allow the bacteria to interact with coiled-coil structures on human cells, e.g., receptors of the C-type lectin superfamily (Beavil et al., 1992).

The M protein family of *S. pyogenes* provides these bacteria with the ability to bind host proteins like IgG, albumin, and fibrinogen. The present data suggest that members of this family can switch between two temperature-dependent states, an active folded state and an inactive unfolded state, and that the switch *in vivo* is triggered by temperature changes close to 37 °C. Thus, temperature fluctuations in the bacterial environment could change the physicochemical surface properties of the bacterium and thereby influence the host–parasite relationship during *S. pyogenes* infections.

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REFERENCES

Åkesson, P., Cooney, J., Kishimoyo, F., & Björck, L. (1990) *Mol. Immunol. 27*, 523–531.
Åkesson, P., Schmidt, K.-H., Cooney, J., & Björck, L. (1994) *Biochem. J. 300*, 877–886.
Åkerström, B., & Björck, L. (1989) *J. Biol. Chem. 264*, 19740–19746.
Åkerström, B., Lindahl, G., Björck, L., & Lindqvist, A. (1992) *J. Immunol. 148*, 3238–3242.
Beavil, A. J., Edmeades, R. L., Gould, H. J., & Sutton, B. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 753–757.
Ben Nasr, A., Wistedt, A., Ringdahl, U., & Sjöbring, U. (1994) *Eur. J. Biochem. 222*, 267–276.
Berge, A., & Sjöbring, U. (1993) *J. Biol. Chem. 268*, 25417–25424.
Cantor, C. R., & Schimmel, P. (1980) *Biophysical chemistry, Part II: Techniques for the study of biological structure and function*, W. H. Freeman and Company, New York.
Cedervall, T., Åkesson, P., Stenberg, L., Herrmann, A., & Åkerström, B. (1995) *Scand. J. Immunol.* (in press).
Clarke, J., & Fersht, A. R. (1993) *Biochemistry 32*, 4322–4329.
Cohen, C., & Parry, D. A. D. (1990) *Proteins: Struct., Funct., Genet. 7*, 1–15.
Conway, J. F., & Parry, D. A. D. (1990) *Int. J. Biol. Macromol. 12*, 328–334.
Cooper, T., & Woody, R. W. (1990) *Biopolymers 30*, 657–676.
Crick, F. H. C. (1953) *Acta Crystallogr. 6*, 689–697.
de Château, M., & Björck, L. (1994) *J. Biol. Chem. 269*, 12147–12151.
Deisenhofer, J. (1981) *Biochemistry 20*, 2361–2370.
Dell, A., Antone, S. M., Gauntt, C. J., Crossley, C. A., Clark, W. A., & Cunningham, M. W. (1991) *Eur. Heart J. 12*, 158–162.
Fietz, M. J., McLaughlan, C. J., Campbell, M. T., & Rogers, G. E. (1993) *J. Cell. Biol. 121*, 855–865.
Fischetti, V. A. (1989) *Clin. Microbiol. Rev. 2*, 285–314.
Fischetti, V. A., Parry, D. A. D., Trus, B. L., Hollingshead, S. K., Scott, J. R., & Manjula, B. N. (1988) *Proteins: Struct., Funct., Genet. 3*, 60–69.
Frick, I.-M., Wikström, M., Forsén, M., Forsén, S., Drakenberg, T., Gomi, H., Sjöbring, U., & Björck, L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8532–8536.
Frick, I.-M., Åkesson, P., Cooney, J., Sjöbring, U., Schmidt, K., Gomi, H., Hattori, S., Tagawa, C., Kishimoto, F., & Björck, L. (1994) *Mol. Microbiol. 12*, 143–151.
Frick, I.-M., Crossin, K. L., Edelman, G. L., & Björck, L. (1995) *EMBO J. 14*, 1674–1679.
Frithz, E., Hedén, L.-O., & Lindahl, G. (1989) *Mol. Microbiol. 3*, 1111–1119.
Gomi, H., Hozumi, T., Hattori, S., Tagawa, C., Kishimoto, F., & Björck, L. (1994) *J. Immunol. 144*, 4046–4052.
Gouda, H., Torigoe, H., Saito, A., Sato, M., Arata, Y., & Shimada, I. (1992) *Biochemistry 31*, 9665–9672.
Gronenborn, A. M., Filpula, D. R., Essig, N. Z., Achari, A., Whitlow, M., Wingfield, P. T., & Clore, G. M. (1991) *Science 253*, 657–661.
Guss, B., Eliasson, M., Olsson, A., Uhlén, M., Frej, A.-K., Jörnvall, H., Flock, J.-I., & Lindberg, M. (1986) *EMBO J. 5*, 1567–1575.
Hanski, E., & Caparon, M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6172–6176.
Heath, D. G., & Cleary, P. P. (1987) *Infect. Immun. 55*, 1233–1238.
Heath, D. G., & Cleary, P. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4741–4745.
Hodges, R. S., Sodek, J., Smillie, L. B., & Jurasek, J. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 299–310.
Hodges, R. S., Semchuk, P. D., Taneja, A. K., Kay, C. M., Parker, J. M. R., & Mant, C. T. (1988) *Peptide Res. 1*, 19–30.
Hodges, R. S., Zhou, N. E., Kay, C. M., & Semchuk, P. D. (1990) *Peptide Res. 3*, 123–137.
Hollingshead, S. K., Fischetti, V. A., & Scott, J. R. (1987) *Infect. Immun. 55*, 3237–3239.
Hosein, B., McCarte, M., & Fischetti, V. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3765–3768.
Janson, H., Hedén, L.-O., Grubb, A., Ruan, M., & Rorsgren, A. (1991) *Infect. Immun. 59*, 119–125.
Kastern, W., Sjöbring, U., & Björck, L. (1992) *J. Biol. Chem. 267*, 12820–12825.
Kehoe, M. A. (1994) *New. Comp. Biochem. 27*, 217–261.
Khandke, K. M., Fairwell, T.,& Manjula, B. N. (1987) *J. Exp. Med. 166*, 151–162.

Khandke, K. M., Fairwell, T., Acharya, A. S., Trus, B. L., & Manjula, B. N. (1988) *J. Biol. Chem. 263*, 5075−5082.
Kraus, W., Ohyama, K., Snyder, D. S., & Beachey, E. H. (1989) *J. Exp. Med. 169*, 481−492.
Kraus, W., Dale, J. B., & Beachey, E. H. (1990) *J. Immunol. 145*, 4089−4093.
Lau, S. Y. M., Taneja, A. K., & Hodges, R. S. (1984) *J. Biol. Chem. 259*, 13253−13261.
Laurent, T. C., & Killander, J. (1964) *J. Chromatogr. 14*, 317−330.
Lee, S.-C., Kim, I.-G., Marekov, L. N., O'Keefe, E. J., Parry, D. A. D., & Steinert, P. M. (1993) *J. Biol. Chem. 268*, 12164−12176.
Lian, L.-Y., Derrick, J. P., Sutcliffe, M. J., Yang, J. C., & Roberts, G. C. K. (1992) *J. Mol. Biol. 228*, 1219−1234.
Lindahl, G., & Åkerström, B. (1989) *Mol. Microbiol. 3*, 239−247.
Lindahl, G., & Stenberg, L. (1990) *Epidemiol. Infect. 105*, 87−93.
Lupas, A., Van Dyke, M., & Stock, J. (1991) *Science 252*, 1162−1164.
Manjula, B. N., Khandke, K. M., Fairwell, T., Relf, W. A., & Sriprakash, K. S. (1991) *J. Protein Chem. 10*, 369−384.
McLachlan, A. D., & Stewart, M. (1975) *J. Mol. Biol. 98*, 293−304.
McLachlan, A. D., & Stewart, M. (1976) *J. Mol. Biol. 103*, 271−298.
Miller, L., Gray, L., Beachey, E., & Kehoe, M. (1988) *J. Biol. Chem. 263*, 5668−5673.
Monera, O., Kay, C. M., & Hodges, R. (1994) *Biochemistry 33*, 3862−3871.
Mouw, A. R., Beachey, E. H., & Burdett, V. (1988) *J. Bacteriol. 170*, 676−684.
O'Toole, P., Stenberg, L., Rissler, M., & Lindahl, G. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8661−8665.
Parry, D. A. D. (1982) *Biosci. Rep. 2*, 1017−1024.
Pheil, W. (1986) in *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., Ed.) pp 349−376, Springer Verlag, Berlin.
Phillips, G. N., Flicker, P. F., Cohen, C., Manjula, B. N., & Fischetti, V. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4689−4693.
Podbielski, A. (1993) *Mol. Gen. Genet. 237*, 287−300.
Retnoningrum, D. S., & Cleary, P. P. (1994) *Infect. Immun. 62*, 2387−2394.
Retnoningrum, D. S., Podbielski, A., & Cleary, P. P. (1993) *J. Immunol. 150*, 2332−2340.
Robbins, J. C., Spanier, J. G., Jones, S. J., Simpson, W. J., & Cleary, P. P. (1987) *J. Bacteriol. 169*, 5633−5640.
Rost, B., & Sander, C. (1993) *J. Mol. Biol. 232*, 584−599.
Rost, B., & Sander, C. (1994) *Proteins: Struct., Funct., Genet. 19*, 55−72.
Rost, B., Casadio, R., Fariselli, P., & Sander, C. (1995) *Protein Sci. 4*, 521−533.
Sauer-Eriksson, A. E., Kleywegt, G. J., Uhlén, M., & Jones, T. A. (1995) *Structure 3*, 265−278.
Schmidt, K.-H., & Wadström, T. (1990) *Zbl. Bakt. 273*, 216−228.
Sreerama, N., & Woody, R. W. (1993) *Anal. Biochem. 209*, 32−44.
Stenberg, L., O'Toole, P. W., Mestecky, J., & Lindahl, G. (1994) *J. Biol. Chem. 269*, 13458−13464.
Stone, D., Sodek, J., Johnson, P., & Smillie, L. B. (1975) in *Proteins of Contractile Systems*, Proceedings of the IX Federation of European Biochemical Societies Meeting (Biro, E. N. A., Ed.) Vol. 31, pp 125−136, North Holland Publishing, Amsterdam.
Stone, G., Sjöbring, U., Björck, L., Sjöquist, J., Barber, C., & Nardella, F. (1989) *J. Immunol. 143*, 565−570.
Swanson, J., Hsu, K. C., & Gotschlich, E. C. (1969) *J. Exp. Med. 130*, 1063−1091.
Uhlén, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L., & Lindberg, M. (1984) *J. Biol. Chem. 259*, 1695−1702.
Vashishtha, A., & Fischetti, V. A. (1993) *J. Immunol. 150*, 4693−4701.
Wikström, M., Sjöbring, U., Kastern, W., Björck, L., Drakenberg, T., & Forsén, S. (1993) *Biochemistry 32*, 3381−3386.
Wikström, M., Drakenberg, T., Forsén, S., Sjöbring, U., & Björck, L. (1994) *Biochemistry 33*, 14011−14017.
Zhou, N. E., Kay, C. M., & Hodges, R. S. (1992a) *Biochemistry 31*, 5739−5746.
Zhou, N. E., Kay, C. M., & Hodges, R. S. (1992b) *J. Biol. Chem. 267*, 2664−2670.
Zhou, N. E., Kay, C. M., Sykes, B. D., & Hodges, R. S. (1993) *Biochemistry 32*, 6190−6197.
Zhou, N. E., Kay, C. M., & Hodges, R. S. (1994) *J. Mol. Biol. 237*, 500−512.

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