C Repeats of the Streptococcal M1 Protein Achieve the Human Serum Albumin Binding Ability by Flanking Regions Which Stabilize the Coiled-Coil Conformation†

Knut Gubbe,‡ Rolf Misselwitz,§,|| Karin Welfle,||,⊥ Werner Reichardt,‡ Karl-Hermann Schmidt,‡,¶ and Heinz Welfle*,§,||

Institute of Experimental Microbiology, University of Jena, Winzerlaer Strasse 10, D-07745 Jena, Germany, Institute of Biochemistry, Medical Faculty (Charité), Humboldt-University, Berlin, Schumannstrasse 20-21, D-10098 Berlin, Germany, and Institute of Biology, Faculty I for Mathematical and Natural Sciences, Humboldt-University, Berlin, Chaussee Strasse 117, D-10115 Berlin, Germany

Received December 5, 1996; Revised Manuscript Received March 20, 1997⊗

ABSTRACT: The M and M-like proteins of *Streptococcus pyogenes* are fibrous cell surface proteins. They have multiple binding sites for several human proteins and are composed of the C-terminal anchor domain, the α-helical coiled-coil domain, and the N-terminal non-coiled-coil domain. The coiled-coil domain of the M1 protein consists of repeat units called B, C, and D and a spacer unit S between B and C. Recombinant fragments A-B-S-C-D, A-B-S, B-S-C, S-C, S-C-D, C-D, and C of the coiled-coil domain were studied by analyzing their secondary structures and binding affinities to human serum albumin (HSA). As shown by circular dichroism, all fragments are in an α-helical conformation. C-D and S-C-D form coiled coils at room temperature and bind below 37 °C with high affinity to HSA. C-D and S-C-D unfold in two steps with T_m values of ∼31 and ∼65 °C; complex formation with HSA increases the unfolding temperatures. B-S-C has a lower α-helical content, a less pronounced coiled-coil conformation, and a reduced thermal stability, binds HSA weaker, and is only slightly stabilized by HSA binding in comparison to C-D and S-C-D. C and S-C are less stable than the other fragments and are not organized as coiled coils showing some features of α-helical single strands only below 20 °C, and binding of HSA was not observed. The results indicate that the formation of coiled-coil structures, supported by flanking D regions and, to a lesser extent also B regions, is essential for the binding of C repeat units to HSA.

M proteins[1] belong to a family of fibrous streptococcal surface proteins. These molecules share a conserved C-terminal region close to the cell surface. The N-terminal half, distal to the cell wall, is highly variable. M proteins protect streptococci against phagocytosis [for reviews see Fischetti (1989) and Kehoe (1994)] and bind host proteins such as immunoglobulins (Heath & Cleary, 1989; Åkesson et al., 1990; Schmidt & Wadström, 1990; Heden & Lindahl, 1993), human serum albumin (HSA) (Schmidt & Wadström, 1990; Schmidt et al., 1993; Retnoningrum & Cleary, 1994), fibrinogen (Kantor, 1965), and fibronectin (Schmidt et al., 1993; Frick et al., 1994). Fibrinogen was shown to mask protective antigenic epitopes on the M protein molecule (Whitnack et al., 1984), whereas HSA was shown to protect already ingested streptococci against intracellular killing (Wagner et al., 1986).

The conserved repetitive C units are responsible for several biological activities of M and M-like proteins. The C units are involved in the binding of HSA to the M protein (Åkesson et al., 1994; Retnoningrum & Cleary, 1994). Furthermore, the binding of complement factor H (Fischetti et al., 1995) and streptococcal adherence to keratinocytes in human skin (Perezcasal et al., 1995) are mediated by the C units, and moreover, antibodies directed against this region are cross-reactive with denatured forms of myosin (Vashishtha & Fischetti, 1993).

M proteins show some structural similarities with the mammalian fibrous proteins tropomyosin, myosin, and keratin (Fischetti, 1989). M proteins are composed of characteristic seven-residue amino acid repeats (a•b•c•d•e•f•g heptads), show a high degree of α-helicity, and have a fibrillar structure (Phillips et al., 1981; Fischetti, 1989; Nilson et al., 1995). They can be modeled as α-helical coiled-coil dimers. The coiled-coil motif is widely observed in biological molecules with different functions (Cohen & Parry, 1990; Adamson et al., 1993; Dohlman et al., 1993). The main forces for the formation and stability of the coiled-coil structures are interactions of hydrophobic amino acids at positions a and d of the a•b•c•d•e•f•g heptad repeat in the interacting chains (Hodges et al., 1972; Zhou et al., 1992). In addition, ion pairs between e and g positions of the heptad repeats of the two α-helical chains contribute to the stability and the orientation of the coiled-coil structure (Zhou et al.,

† K.G., W.R., and K.-H.S. were supported by DFG Grant Schm 877/1-2.

* To whom correspondence should be addressed.

‡ University of Jena.

¶ Present address: Institute for Medical Microbiology.

§ Institute of Biochemistry, Humboldt-University, Berlin.

|| c/o Max-Delbrück-Centre for Molecular Medicine, Robert-Rössle-Strasse 10, D-13122 Berlin, Germany.

⊥ Institute of Biology, Humboldt-University, Berlin.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

[1] Abbreviations: M1 protein, M family surface protein from *Streptococcus pyogenes* strain 40/58; A-B-S-C-D, full length coiled-coil domain of the M1 protein composed of regions A, B, S, C, and D; A-B-S, B-S-C, S-C-D, S-C, C-D, and C, M1 protein fragments containing the indicated regions; HSA, human serum albumin; Ig, immunoglobulin; TFE, 2,2,2-trifluoroethanol; PBS, phosphate-buffered saline; IPTG, isopropyl β-D-thiogalactopyranoside; FPLC, fast protein liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism; $[\Theta]_{222}$, ellipticity at 222 nm; T_m (degrees Celsius), half-transition temperature.

S0006-2960(96)02991-1 CCC: $14.00

1994; Monera et al., 1994).

The interaction of M family proteins with IgG is temperature-dependent. This was shown for proteins H and Arp (Åkerström et al., 1992) and more recently for proteins Sir and M1 (Cedervall et al., 1995). The IgG binding correlates with a temperature-dependent coiled-coil dimerization (Åkerström et al., 1992). Furthermore, binding of IgG, HSA, or IgG plus HSA increases the thermal stability of proteins H and M1 and the M1 fragment S-C3, containing the S and C regions and a small part of the D region (Nilson et al., 1995).

In a preliminary study, we cloned and expressed several fragments of the M1 and M3 proteins (Reichardt et al., 1995). Surprisingly, fragments consisting of three (M1) or two (M3) C repeat units only did not bind to HSA. This was in apparent conflict with recent results that identified C units as the elements responsible for M protein binding to HSA (Åkesson et al., 1994; Retnoningrum & Cleary, 1994) and encouraged us to examine in detail the role of the C repeat region for the binding of HSA. Here, we produced M1 protein fragments of different sizes composed of C repeats and characteristic flanking sequences and studied their binding affinity to HSA and their conformation and stability. HSA binding was tested at different temperatures, and the results were correlated with temperature-induced structural changes of M1 fragments monitored by circular dichroic measurements in the absence or presence of HSA.

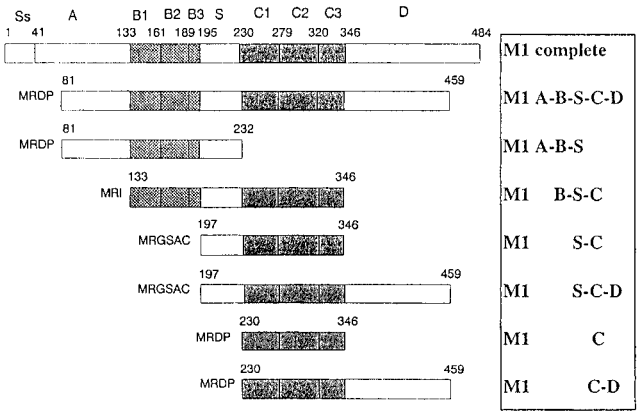

FIGURE 1: Schematic representation of constructed M1 fragments. The numbering scheme is based on the M1 primary structure published by Åkesson et al. (1994). The complete M1 sequence composed of amino acids 1−484 is divided into 10 regions symbolized by segments of the bar. M1 starts N-terminally with the Ss region, followed by the A region, the B region composed of three repeat units (B1, B2, and B3), a spacer region called S, and the C region composed of three repeat units (C1, C2, and C3), and ends C-terminally with the D region. Fragments are named by letter abbreviations given in the frame. The largest fragment, A-B-S-C-D, extends from amino acid 81 to 459 of the complete M1 sequence, enclosing part of region A, region B with three units, spacer region S, region C with three units, and a part of region D. Heterologous amino acids MRDP derived from the pQE vector are indicated in the single-letter code at the N terminus. The other fragments are named and characterized accordingly.

MATERIALS AND METHODS

Strains. Type M1 *Streptococcus pyogenes* strain 40/58 from the strain collection of the Streptococcal Laboratory of the University of Jena (Germany) was used for the preparation of the entire M1 protein and for isolation of chromosomal DNA. Strain 40/58 was originally provided by J. Havlicek of the WHO Streptococcal Reference Center (Prague, Czechia) and was renamed strain AP1 by others. *Escherichia coli* strains Epicurian XL1-Blue MRF′Kan (Stratagene, La Jolla, CA) and M15 (pREP4) (QIAGEN, Hilden, Germany) were used for cloning.

PCR and Cloning Procedures. The following primers were deduced from the *emm1* sequences published recently (Harbaugh et al., 1993; Åkesson et al., 1994) to synthesize PCR fragments of the *emm1* gene including synthetic restriction sites at the 5′ ends: forward primers M1A-f AAG CGA GCT CAG AGG ATC CAA TGG AAG TTG CAG GAA GAG AT (with a *Bam*HI site), M1B-f GGG ATA GAC GGA TCC TTG AAA AAG AG (with a *Bam*HI site), M1S-f GAA GCA TGC ACT AGA GAA CAA GAG CTC AAT CGT (with a *Sph*I site), and M1C-f CCT GGA TCC AAA ACT TGA GGA AGA AA (with a *Bam*HI site) and reverse primers M1S-r TGC GGT CGA CTG CAG CCC GGG TTA CTC AAG TTT TGC TTT TTC (with a *Pst*I site), M1C-r CTT GGA TCC TTA AGT TTT TCA AGA GCA GCT AAT TT (with a *Bam*HI site), and M1D-r GGA AGA TCT GCA GTC GAC TTA TGG GTT AGC TGT TTC ACC TGT TGA (with a *Pst*I site).

The sequences of the fragments and their correlation to the M1 sequence (Åkesson et al., 1994) were established in the following manner. PCR fragment A-B-S-C-D (Figure 1) was synthesized using primers M1A-f and M1D-r and chromosomal DNA of strain M1 40/58 as a template. The amplified DNA fragment was ligated into the cloning vector pCR-ScriptTM SK(+) from Stratagene and subcloned in *E. coli* strain Epicurian XL1-Blue MRF′Kan. The insert was partially sequenced using primers T3 and T7 from the pCR-Script vector kit. The 5′ part and the converted 3′ part of the sequence were identical to that of Åkesson et al. (1994). Plasmid DNA of this clone was used as a template for the generation of PCR fragments for the other M1 fragments shown in Figure 1. The truncated *emm1* fragments were cut out with appropriate restriction enzymes and ligated into one of the pQE-50, pQE-51, or pQE-52 expression vectors from QIAGEN.

For the expression of M1 fragments B-S-C, S-C, and C, the PCR fragments were produced with the reverse primer M1C-r. The cloned PCR fragments were digested with *Hind*III before ligation in pQE vectors. The *Hind*III restriction site in the *emm1* DNA sequence is located at position 1034 at the end of the encoding C repeat (C3, see Figure 1). The constructs result in the expression of M1 fragments that start at the N terminus with heterologous amino acids derived from the pQE vectors (Figure 1).

After transformation in *E. coli* strain M15 (pREP4), clones were screened for expression. Expressed M1 fragments were identified with anti-M1 rabbit sera or with peroxidase-labeled HSA by Western blot analysis as described previously (Schmidt et al., 1993).

Protein Expression and Purification. Large scale expression of M1 fragments in pQE vectors was performed according to the manufacturer's protocols. *E. coli* transformants were grown in 100 mL for 6 h and for 3 h more after addition of IPTG. The cells were collected, washed, and suspended in 10 mL of 0.02 M Tris-HCl buffer at pH 7.2. After sonification of the cells, the lysates were centrifuged, and the clear supernatants were fractionated by FPLC ion exchange chromatography on a Q-Resource column (Pharmacia, Uppsala, Sweden) in 0.02 M sodium phosphate buffer

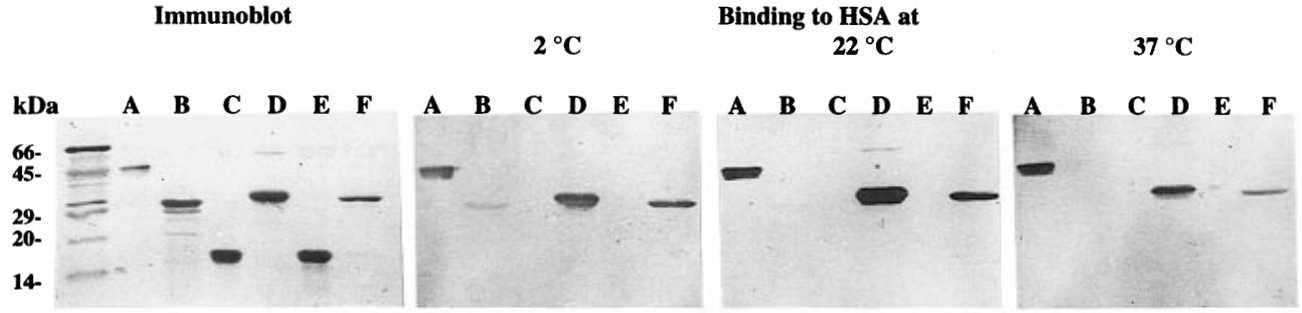

FIGURE 2: Western blot analysis and temperature-dependent binding of HSA to M1 protein fragments. Protein fragments were separated by SDS−PAGE and incubated with purified M1 antibodies (immunoblot on the left) after transfer to nitrocellulose membranes. To assay for binding of HSA the membranes were probed at 2, 22, and 37 °C with peroxidase-labeled HSA: lane A, A-B-S-C-D; lane B, B-S-C; lane C, S-C; lane D, S-C-D; lane E, C; and lane F, C-D.

at pH 7.2, applying a linear salt gradient from 0 to 1 M NaCl. Fractions with affinity to peroxidase-labeled HSA and/or anti-M1 antiserum were collected, dialyzed, and further separated on an S-Resource column (Pharmacia) equilibrated with 0.02 M sodium citrate/sodium phosphate buffer at pH 6.0, applying again a linear salt gradient from 0 to 1 M NaCl.

Western Blot Analysis. The purity of the M1 fragments was analyzed by SDS−PAGE. The relation to the M1 protein was confirmed by Western blotting on nitrocellulose membranes with affinity-purified and peroxidase-labeled anti-M1 C antibodies from rabbit. The antibodies were isolated by affinity chromatography of rabbit anti-M1 protein serum on fragment C immobilized to CNBr-activated Sepharose.

HSA Binding Assay. The concentrations of the fragments were adjusted to 100 μg/mL. After SDS−PAGE, the proteins were electrotransferred to nitrocellulose membranes. Filters were blocked with skim milk in PBS containing 0.02% Tween 20 and then incubated for 2 h at different temperatures (2, 22, and 37 °C) with peroxidase-labeled HSA. The blots were visualized with 3-amino-9-ethylcarbazole as described recently (Schmidt et al., 1993).

Protein Concentration Determination. Before spectroscopic and FPLC measurements, the M1 fragments were dialyzed exhaustively against 10 mM sodium phosphate buffer, (pH 7.4) or against 10 mM sodium phosphate buffer (pH 7.4) and 0.15 M NaCl. The studied fragments of the M1 protein, with the exception of B-S-C, do not contain aromatic amino acids. Therefore, the protein concentrations were determined from amino acid analyses after hydrolysis in the gas phase with 6 N HCl for 24 h according to Meltzer et al. (1987). Protein samples at pH 2.0 were prepared in 10 mM HCl. Protein samples containing ultrapure urea (ICN Biochemicals, Cleveland, OH) or trifluorethanol (TFE) (Aldrich, Steinheim, Germany) were prepared in 10 mM sodium phosphate buffer at pH 7.4 by adding a 10 M urea solution in buffer or TFE aliquots.

FPLC Measurements. Analytical gel filtration was performed at 20 °C on a FPLC Superose 12 HR 10/30 column (Pharmacia). Protein samples of about 0.2 mg/mL in 10 mM sodium phosphate buffer (pH 7.4) and 0.15 M NaCl were applied to the equilibrated column. The column was calibrated with proteins of the Pharmacia gel calibration kits. Frictional ratios, f/f_{min}, were estimated from the ratio r_s/r_0, where r_s is the Stokes radius and r_0 is the radius of the anhydrous sphere. r_0 was calculated using the equation $r_0 = (3Mv/4\pi N)^{1/3}$, where M is the molar mass, v is the partial specific volume of 0.72 cm^3 g^{-1}, and N is Avogadro's number (6.02×10^{23}).

Circular Dichroism. CD measurements were performed with a Jasco J-720 spectropolarimeter (Jasco Instruments S. A., Japan) as described recently (Misselwitz et al., 1995). The instrument was equipped with a thermostated cell holder and a temperature control system (Neslab, USA). Far-ultraviolet CD spectra of M1 fragments at various protein concentrations were measured in cuvettes with 0.010, 0.10, or 1.0 cm path lengths at 2 °C in 10 mM sodium phosphate buffer at pH 7.4 with or without 0.15 M NaCl. Molar ellipticities of M1 fragments are expressed as mean residue molar ellipticities [Θ] (deg cm^2 dmol^{-1}) using mean residue molecular weights of 116.0 for B-S-C, 111.5 for S-C-D, 111.7 for C-D, 115.1 for S-C, and 116.2 for C.

Thermal Unfolding Studies. Temperature-dependent conformational transitions were monitored by measuring changes of the circular dichroism at 222 nm. The unfolding experiments were performed in cells with a 0.1 cm path length at a protein concentration of 4.2 μM and a heating rate of 20 °C h^{-1}. Effects of HSA on the thermal stability of M1 fragments were studied at the same HSA and fragment concentration of 4.2 μM. The final temperature-induced transition curves were estimated by subtracting the unfolding curve of HSA from the unfolding curves of the mixtures of fragments and HSA, measured under identical experimental conditions. The half-transition temperatures (T_m^1 and T_m^2) were determined using a program based on a nonlinear regression analysis (written by O. Ristau).

RESULTS

Expression of M1 Protein Fragments and Their Binding to Human Serum Albumin. Seven M1 fragments used in this study are schematically shown in Figure 1. The fragments were obtained as fusion proteins containing three to six heterologous N-terminal amino acids as indicated in Figure 1. The basic structure of the M1 molecule was related to that published by Åkesson et al. (1994).

The fragments were purified by ion exchange chromatography and tested by Western blotting for their ability to bind HSA (Figure 2). Fragment C consisting of three repeats of the C repeat unit does not bind HSA at 22 °C. A-B-S-C-D, S-C-D, and C-D, however, containing C regions flanked at the C-terminal side by sequences of the D region, bind to HSA. B-S-C, flanked at the N-terminal side by sequences of the B and S regions, shows a very weak but still clearly detectable affinity to HSA. Fragment A-B-S, lacking sequences of the C repeat region, does not interact with HSA (not shown).

Table 1: Physicochemical Properties of M1 Protein Fragments

property	S-C-D	C-D	B-S-C	S-C	C
number of amino acids	265	233	217	156	121
molar mass (g mol^{-1})[a]	29 537	26 022	25 177	17 958	14 058
Stokes radius r_s (Å)[b]	53.5	48.7	33.2	30.0	28.2
frictional ratio f/f_{min}	2.09[d]	1.98[d]	1.72[c]	1.74[c]	1.77[c]

[a] Calculated from the amino acid composition. [b] The Stokes radii were estimated by gel chromatography at 20 °C. [c] Calculated assuming a monomeric state at 20 °C. [d] Dimeric state at 20 °C.

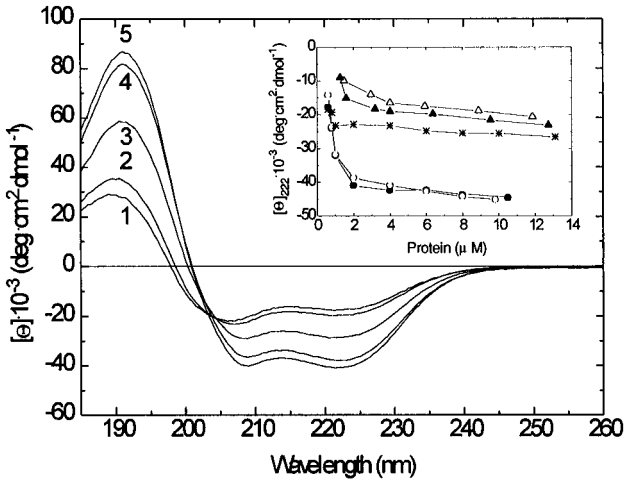

FIGURE 3: Spectroscopic characterization of M1 fragments. Far-UV CD spectra of C (1), S-C (2), B-S-C (3), C-D (4) and S-C-D (5) were measured at 2 °C in 10 mM sodium phosphate buffer at pH 7.4 and at a protein concentration of 4.2 μM. (Inset) Protein concentration dependence of the mean molar residue ellipticity at 222 nm ([Θ]$_{222}$) for C (Δ), S-C (▲), B-S-C (*), C-D (○) and S-C-D (●), measured in 10 mM sodium phosphate buffer (pH 7.4) and 0.15 M NaCl at 2 °C.

Gel Chromatography of M1 Fragments. According to their elution behavior at 20 °C on Superose 12, the M1 fragments can be roughly divided into two groups, with S-C-D and C-D eluting very fast, and B-S-C, S-C, and C eluting moderately faster in comparison to globular reference proteins. Stokes radii, r_s, and frictional ratios, f/f_{min}, were determined (Table 1). Frictional ratios of about 2.0 were obtained for S-C-D and C-D, assuming a dimeric state of the molecules, whereas values of about 1.75 were found for B-S-C, S-C, and C, which were considered to be monomeric in the calculations. Thus, according to these values, especially S-C-D and C-D and, to a smaller extent, also the other fragments have elongated structures (Cantor & Schimmel, 1980).

CD Spectroscopy of M1 Fragments. The far-ultraviolet CD spectra of M1 fragments were measured at 2 °C and are shown in Figure 3. Because of the concentration dependence of the ellipticity (Figure 3, inset), the CD spectra of all fragments were measured at a protein concentration of 4.2 μM as applied by others for the intact M1 protein and the S-C3 fragment (Nilson et al., 1995) to obtain immediately comparable data.

The spectra of all M1 fragments are characterized by double minima at 222 and 208 nm and a maximum at about 192 nm. The large negative [Θ]$_{222}$ values (Table 2) indicate a high helix content. On the basis of a computed [Θ]$_{222}$ value of −39 500 deg cm^2 dmol^{-1} for a helix of infinite length (Chen et al., 1974), fragments S-C-D and C-D with [Θ]$_{222}$ values of −40 600 and −37 700 deg cm^2 dmol^{-1} have

Table 2: Structural Characterization of M1 Protein Fragments

	CD spectra[a] (deg cm^2 dmol^{-1})			transition temperatures[b] (°C)	
fragments	[Θ]$_{222}$	[Θ]$_{222}$/[Θ]$_{208.5}$	[Θ]$_{222}$/[Θ]$_{207}$	T_m^1	T_m^2
S-C-D[c]	−40 630	1.04		31.3	64.8
S-C-D[d]	−46 080	0.93			
S-C-D[e]				30.9	67.6
S-C-D/HSA[f]				35.7	69.4
C-D[c]	−37 770	1.03		31.1	66.1
C-D[d]	−41 910	0.93			
C-D[g]	−16 750		0.76		
C-D/HSA[f]				36.6	67.9
B-S-C[c]	−28 140	1.00			42.8
B-S-C[d]	−33 530	0.95			
B-S-C/HSA[f]					43.5
S-C[c]	−19 330		0.86		
S-C[d]	−27 180	0.95			
C[c]	−16 590		0.80		
C[d]	−29 050	0.93			

[a] CD spectra were recorded at 2 °C and with 4.2 μM protein. [b] Transition curves were obtained measuring changes of the ellipticity at 222 nm in 10 mM sodium phosphate buffer (pH 7.4), 0.15 M NaCl, and 4.2 μM protein. [c] 10 mM sodium phosphate (pH 7.4) and 0.15 M NaCl. [d] 10 mM sodium phosphate (pH 7.4) and 50% (v/v) TFE. [e] Buffer in the presence of 5 mM dithiothreitol. [f] M1 fragment/HSA complexes were prepared by mixing of equimolar amounts. [g] 10 mM HCl (pH 2.0).

a formal helix content of 103 and 96%, respectively. The [Θ]$_{222}$ values measured for B-S-C, S-C, and C are about −28 000, −19 000, and −17 000 deg cm^2 dmol^{-1}, respectively. These [Θ]$_{222}$ values indicate a lower α-helix content of B-S-C, S-C, and C in comparison to that of S-C-D and C-D.

The position of the $\pi-\pi^*$ transition, polarized parallel to the helix axis, is located at 208.5 nm for S-C-D, C-D, and B-S-C and is blue shifted to 207 nm for S-C and C. Mean residue molar ellipticity ratios [Θ]$_{222}$/[Θ]$_{208.5}$ and [Θ]$_{222}$/[Θ]$_{207}$ are informative for dimeric coiled coils and helical, noninteracting single strands. These values are in the range of 1.04−0.80 (Table 2). Values greater than 1.0, estimated for S-C-D and C-D, are similar to that observed recently for dimeric coiled-coil structures (Lau et al., 1984; Hodges et al., 1988; Zhou et al., 1992). Ratios of about 0.83, as found for S-C and C, are characteristic for α-helical, noninteracting single strands, with ellipticities generally more negative at 207 nm than at 222 nm (Cooper & Woody, 1990; Zhou et al., 1993). The ratio of 1.0 found for B-S-C points to an intermediate conformation.

The protein concentration dependence of the negative ellipticities, measured in 10 mM sodium phosphate buffer (pH 7.4) and 0.15 M NaCl, and at 2 °C, varies for the individual fragments as shown in the inset of Figure 3. For S-C-D and C-D (Figure 3, inset, lower two curves), pronounced changes are observed. [Θ]$_{222}$ and the ratio [Θ]$_{222}$/[Θ]$_{208.5}$ increase from 1 to 2 μM protein, suggesting a concentration-dependent shift of the monomer ↔ dimer equilibrium toward dimeric coiled-coil structures.

In Figure 4, far-UV CD spectra of M1 fragment C-D are shown, measured at different temperatures and pH values and in the presence of urea. Comparable results were obtained for the other M1 fragments (data not shown). At 90 °C (Figure 4, spectrum 3) and in 4.5 M urea (Figure 4, spectrum 4), the spectra are very similar. Further denaturant-induced spectral changes were observed up to 8 M urea.

Lowering the pH to 2.0 (Figure 4, spectrum 5) leads to a decrease of [Θ]$_{222}$ and of the [Θ]$_{222}$/[Θ]$_{207}$ ratio but not to

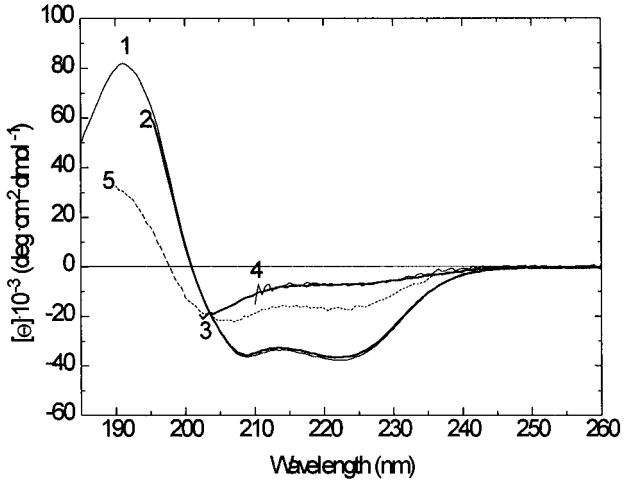

FIGURE 4: Far-UV CD spectra of M1 fragment C-D at various solvent conditions. Circular dichroism spectra were measured at a protein concentration of 4.2 μM in 10 mM sodium phosphate buffer at pH 7.4 (spectra 1−4) and in 10 mM HCl at pH 2.0 (spectrum 5): spectrum 1, 2 °C; spectrum 2, buffer with 0.15 M NaCl at 2 °C; spectrum 3, buffer with 0.15 M NaCl at 90 °C; spectrum 4, buffer with 4.5 M urea at 2 °C; and spectrum 5, 2 °C.

complete unfolding. A $[\Theta]_{222}$ value of about −17 000 deg cm^2 dmol^{-1} indicates a rather high residual amount of α-helical secondary structure (Table 2).

Effects of the ionic strength on the conformation of the M1 fragments are small, as exemplified by the spectra of C-D with and without 0.15 M NaCl (Figure 4, spectra 1 and 2).

CD Measurements in Trifluoroethanol. TFE was widely used as a structure-inducing cosolvent (Sönnichsen et al., 1992). Through the study of coiled-coil model peptides, a significant decrease of the ellipticity ratio $[\Theta]_{222}/[\Theta]_{207}$ was observed in the presence of TFE. This indicates the transition of dimeric α-helical coiled coils to α-helical single strands by disruption of hydrophobic interactions between the two chains (Lau et al., 1984; Cooper & Woody, 1990).

In Figure 5, the CD spectra of fragments C, S-C, C-D, and S-C-D are shown. The spectra were recorded at 2 °C in the absence (full lines) and in the presence of 50% (v/v) TFE (dashed lines). TFE increases the negative ellipticities in the 200−230 nm range. The zero transition points of C (Figure 5A) and S-C (Figure 5B) shift from 197−198 to 201 nm. TFE reduces the $[\Theta]_{222}/[\Theta]_{208.5}$ ratios of S-C-D and C-D from 1.04 to 0.95−0.93. The 207 nm CD band of C and S-C shifts slightly to 208.5 nm, and the $[\Theta]_{222}/[\Theta]_{207}$ ratios of 0.80−0.86 are increased to $[\Theta]_{222}/[\Theta]_{208.5}$ ratios of 0.93−0.95 (Table 2). The 208.5 nm CD bands of S-C-D and C-D do not change their positions. As expected, coiled-coil structures of the M1 fragments are converted into single strands with high α-helicity in the presence of TFE.

Temperature-Induced Structural Changes of M1 Fragments Monitored by Circular Dichroism. CD spectra in the 190−260 nm wavelength range were measured as a function of temperature. The temperature-induced spectral effects are almost completely reversible for the studied M1 fragments. Furthermore, CD spectra of B-S-C, C-D, and S-C-D go through a well-defined isodichroic point at about 203 nm.

Ellipticity versus temperature recordings of C, S-C, B-S-C, C-D, and S-C-D are shown in Figure 6 (full lines). The fragments can be arranged in the order C ≈ S-C < B-S-C < C-D ≈ S-C-D according to increasing thermal stability.

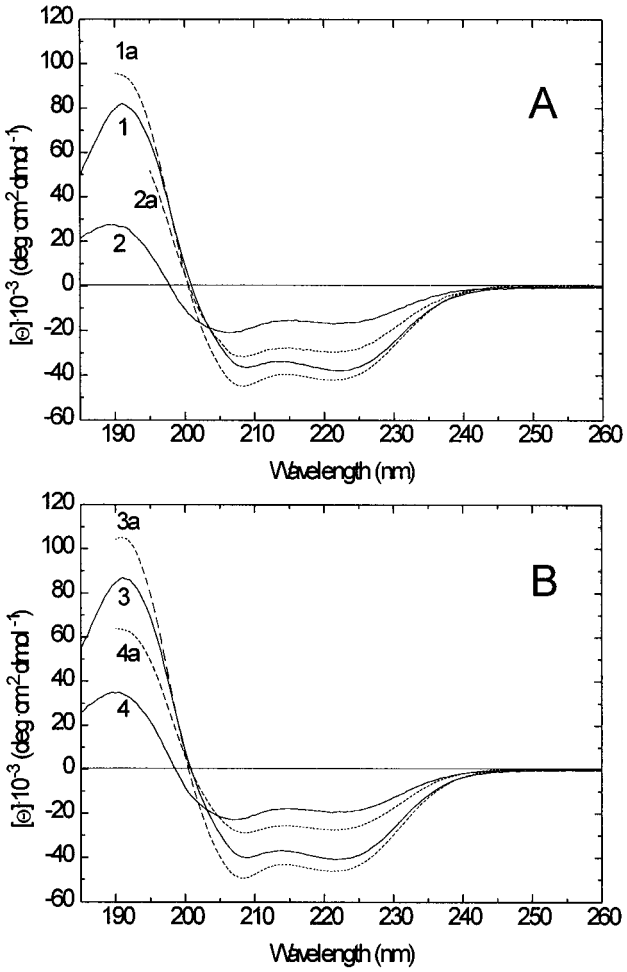

FIGURE 5: Effect of trifluoroethanol on the circular dichroic spectra of M1 fragments. CD spectra in the peptide region of C and C-D (A) and S-C and S-C-D (B) were measured in the absence (1, C-D; 2, C; 3, S-C-D; and 4, S-C) and in the presence of 50% (v/v) TFE (1a, C-D; 2a, C; 3a, S-C-D; and 4a, S-C) at 2 °C in 10 mM sodium phosphate buffer, at pH 7.4.

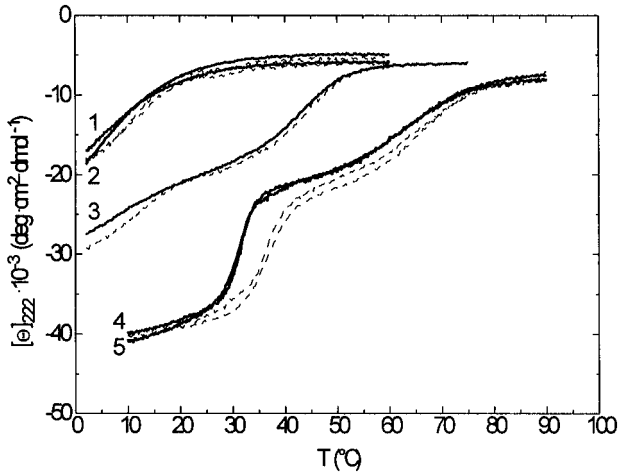

FIGURE 6: Thermal stability of M1 fragments. The ellipticity [Θ] was monitored at 222 nm as a function of temperature in 10 mM sodium phosphate buffer (pH 7.4) and 0.15 M NaCl at a protein concentration of 4.2 μM, without (full lines) and in the presence (dotted lines) of HSA. The ellipticities measured in the presence of HSA were corrected for the contribution of HSA: (1) C, (2) S-C, (3) B-S-C, (4) C-D, and (5) S-C-D.

The temperature dependence of C and S-C (Figure 6, full lines 1 and 2) is very similar and characterized by an almost entire unfolding at about 30 °C. The $[\Theta]_{222}$ values of C

and S-C do not reach low-temperature plateaus, and the S region does not significantly increase the thermal stability of the C region.

The melting behavior of C-D and S-C-D (Figure 6, full lines 4 and 5) is very similar and characterized by two well-separated thermal transitions. The $[\Theta]_{222}$ values have well-defined low-temperature plateaus. The first transition sets in at about 25 °C. An intermediate plateau is reached at about 45 °C where $[\Theta]_{222}$ is reduced to approximately $^1/_2$ of the low-temperature plateau. The second melting step starts at about 55 °C and reaches the high-temperature plateau at about 85 °C. The first transition has a higher cooperativity than the second transition. An S-C-D preparation reduced with 5 mM dithiothreitol shows characteristics of the unfolding curve similar to those of the partially oxidized sample. T_m values of the transitions are summarized in Table 2.

The melting curve of B-S-C (Figure 6, full line 3) indicates a very broad transition at low temperatures. A second somewhat more pronounced high-temperature transition is observed. In comparison to those of C and S-C, the low-temperature transition of B-S-C is shifted to a higher temperature, but the effect of the B region on the unfolding behavior is much smaller than that of the D region.

Effect of Human Serum Albumin on the Thermal Stability of M1 Fragments. The influence of equimolar concentrations of HSA on the thermal stability of M1 fragments was monitored by measuring changes of the ellipticity at 222 nm (Figure 6, dashed lines). The melting curves of the M1 fragment−HSA complexes were corrected by subtraction of the melting curve of HSA recorded in the absence of M1 fragments.

In the presence of HSA, the thermal stability of fragments C and S-C is practically unchanged (Figure 6, dashed lines 1 and 2) as expected for fragments which do not interact with HSA (Figure 2). HSA induces a small increase of the stability of the low-temperature conformation but not of the high-temperature conformation of B-S-C (Figure 6, dashed line 3), consistent with the Western blot analysis described earlier (Figure 2). HSA has pronounced effects on the unfolding behavior of C-D or S-C-D (Figure 6, dashed lines 4 and 5). The transition temperatures T_m of the low- and high-temperature conformations of both fragments increased for about 5 °C (Table 2) and correlate with a strong binding of HSA to C-D and S-C-D at 2, 22, and 37 °C (Figure 2).

DISCUSSION

Here, we demonstrate that the conformation of the C repeat region of the streptococcal M1 protein of the type 1 strain 40/58 plays an important role in the interaction with HSA. Through the study of several M1 fragments, it is shown that isolated C repeat regions cannot bind but have to form dimeric coiled-coil structures as a prerequisite for HSA binding. Isolated C repeats without flanking sequences as represented by fragment C form α-helical single strands. Flanking sequences of the B region (fragment B-S-C**)** and, more efficiently, of the D region (fragments S-C-D and C-D) promote the formation of dimeric coiled-coil structures and restore the affinity to HSA. Spacer region S does not influence secondary structure, HSA binding, and thermal stability. HSA binding further stabilizes the dimeric conformation. Thus, the C repeat region of the M1 protein by itself is essential but not sufficient for binding to HSA since flanking regions have to stabilize its dimeric coiled-coil conformation.

Circular dichroism studies on M6, H, M1, and M1 fragment S-C3 showed a high α-helix content and a coiled-coil structure of these proteins (Phillips et al., 1981; Nilson et al., 1995). All M1 fragments investigated here are also, at least at low temperatures, in an α-helical conformation. However, only fragments C-D and S-C-D, and partially B-S-C, are folded as two-stranded coiled coils. The smaller fragments, S-C and C, are less α-helical, and the CD spectra are similar to that described by Cooper and Woody (1990) and Zhou et al. (1993) for noninteracting α-helical single strands. The frictional ratios f/f_{min} suggest that D region-containing M1 fragments S-C-D and C-D are structured in a more elongated and rigid conformation than B-S-C, S-C, and C. An elongated, rigid dimeric coiled-coil conformation was described recently for proteins H, Arp, and intact M1 (Åkerström et al., 1992; Nilson et al., 1995).

Intact M1 protein unfolds thermally in a complex non-two-state manner (Nilson et al., 1995). Thermal unfolding of M1 fragments C-D and S-C-D, studied here, is characterized by two transitions with T_m values of ∼31 and ∼65 °C for the first and the second transition, respectively. It is tempting to speculate that during the first transition the dimeric coiled coils dissociate which is accompanied by the unfolding of structural elements of the monomeric C repeat region (and S region). Possibly, the second transition is connected with the unfolding of structural elements of the D region.

M1 fragments C and S-C, lacking the D region, have a drastically reduced thermal stability and are almost completely unfolded at room temperature. Interestingly, the thermal stability of the S-C fragment studied here is distinctly lower than that of the S-C3 fragment recently described by Nilson et al. (1995) which unfolds in a "two-state" transition with a T_m value of about 17 °C. This discrepancy can probably be explained by the presence in S-C3 of 11 additional C-terminal amino acid residues derived from the D region (Åkesson et al., 1994).

In a preliminary study (Reichardt et al., 1995) and here, we show that C repeats without flanking regions do not bind HSA at room temperature. CD data indicate that fragment C is unfolded at room temperature. Below room temperature, fragment C partially refolds, but this is not connected with a measurable HSA binding. On the other hand, fragments C-D and S-C-D show strong HSA binding up to 37 °C correlating with spectral features that indicate the existence of coiled-coil structures. Furthermore, the thermal stability of C-D and S-C-D fragments was increased by the binding of HSA. Ligand-induced stabilization of protein H and intact protein M1 was observed recently in the presence of HSA, IgG, and both HSA and IgG (Nilson et al., 1995).

The stability and conformation of M1 fragments are studied here by measuring spectroscopic properties of their solutions. *In vivo*, however, protein M1 is bound to the cell wall by a C-terminal anchor domain. This might effect its stability. We can only speculate about the structure and stability of intact M1 molecules, bound to the cell wall. Possibly, the dimeric coiled-coil conformation of M1 is more stable than that of soluble fragments. This might be achieved by a close vicinity of the chains anchored in the wall. Thus, even at 37 °C, the physiological temperature, the existence

of coiled-coil dimers is expected. *In vitro* blotting was performed with immobilized protein. Since immobilization is not defined, this may not really mimic properties of cell surface-bound protein, but it demonstrates the conservation of functional properties under artificial conditions.

Our data confirm the results of Nilson et al. (1995) on the structure and stability of the M1 protein and extend these data detailing the functional role of the conformation of the C repeat units. There is a minor difference between earlier data demonstrating HSA binding by fragment S-C3 (Åkesson et al., 1994) and our finding that fragment S-C does not bind HSA. This might be an apparent contradiction since S-C3 contains a short C-terminal segment of 11 amino acids missing in fragment S-C. The additional segment is derived from the D region and, as discussed already with regard to the higher thermal stability of S-C3 in comparison to that of S-C, possibly responsible for the observed differences. This would underline the importance of the D region for the conformation of C repeats.

ACKNOWLEDGMENT

We thank Dr. R. Kraft for performing amino acid analyses, Mrs. B. Kannen and Mrs. K. Lauer for skillful technical assistance, and Dr. U. Heinemann for critical reading of the manuscript.

REFERENCES

Adamson, J. G., Zhou, N. E., & Hodges, R. S. (1993) *Curr. Opin. Biotechnol. 4*, 428−437.

Åkerström, B., Lindahl, G., Björck, L., & Lindqvist, A. (1992) *J. Immunol. 148*, 3238−3243.

Åkesson, P., Conney, J., Kishimoto, F., & Björck, L. (1990) *Mol. Immunol. 27*, 523−531.

Åkesson, P., Schmidt, K.-H., Cooney, J., & Björck. L. (1994) *Biochem. J. 300*, 877−886.

Cantor, C. R., & Schimmel, P. (1980) *Biophysical Chemistry, Part II: Techniques for 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. 42*, 433−441.

Chen, Y.-H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350−3359.

Cohen, C., & Parry, D. A. D. (1990) *Proteins: Struct., Funct., Genet. 7*, 1−15.

Cooper, T. M., & Woody, R. W. (1990) *Biopolymers 30*, 657−676.

Dohlman, J. G., Lupas, A., & Carson, M. (1993) *Biochem. Biophys. Res. Commun. 195*, 686−696.

Fischetti, V. A. (1989) *Clin. Microbiol. Rev. 2*, 285−314.

Fischetti, V. A., Horstmann, R. D., & Pancholi, V. (1995) *Infect. Immun. 63*, 149−153.

Frick, I.-M., Åkesson, P., Cooney, J., Sjöbring, U., Schmidt, K.-H., Gomi, H., Hattori, S., Tagawa, C., Kishimoto, F., & Björck, L. (1994) *Mol. Microbiol. 12*, 143−151.

Harbaugh, M. P., Podbielski, A., Hugl, S., & Cleary, P. P. (1993) *Mol. Microbiol. 8*, 981−991.

Heath, D. G., & Cleary, P. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4741−4745.

Heden, L.-O., & Lindahl, G. (1993) *J. Gen. Microbiol. 139*, 2067−2074.

Hodges, R. S., Sodeck, J., Smillie, L. B., & Jurasek, L. (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) *Pept. Res. 1*, 19−30.

Kantor, F. S. (1965) *J. Exp. Med. 121*, 848−859.

Kehoe, M. A. (1994) in *Bacterial Cell Wall* (Ghuysen, J. M., & Hakenbeck, R., Eds.) pp 217−261, Elsevier Science B. V., Amsterdam.

Lau, S. Y. M., Taneja, A. K., & Hodges, R. S. (1984) *J. Biol. Chem. 259*, 13253−13261.

Meltzer, N. M., Tous, G. L., Gruber, S., & Stein, S. (1987) *Anal. Biochem. 169*, 356−361.

Misselwitz, R., Hausdorf, G., Welfle, K., Höhne, W. E., & Welfle, H. (1995) *Biochim. Biophys. Acta 1250*, 9−18.

Monera, O. D., Kay, C. M., & Hodges, R. S. (1994) *Biochemistry 33*, 3862−3871.

Nilson, B. H. K., Frick, I.-M., Åkesson, P., Forsen, S., Björck, L., Åkerström, B., & Wikström, M. (1995) *Biochemistry 34*, 13688−13698.

Perezcasal, J., Okada, N., Caparon, M. G., & Scott, J. R. (1995) *Mol. Microbiol. 15*, 907−916.

Phillips, G. N., Jr., Flicker, P. F., Cohen, C., Manjula, B. N., & Fischetti, V. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4689−4693.

Reichardt, W., Gubbe, K., & Schmidt, K.-H. (1995) in *Genetics of Streptococci, Enterococci and Lactococci* (Ferretti, J. J., Gilmore, M. S., Klaenhammer, T. R., & Brown, F., Eds.) Vol. 85, pp 179−182, Karger, Basel.

Retnoningrum, D. S., & Cleary, P. P. (1994) *Infect. Immun. 62*, 2387−2394.

Schmidt, K.-H., & Wadström, T. (1990) *Zbl. Bakt. 273*, 216−228.

Schmidt, K.-H., Mann, K., Cooney, J., & Köhler, W. (1993) *FEMS Immunol. Med. Microbiol. 7*, 135−145.

Sönnichsen, F. D., Van Eyk, J. E., Hodges, R. S., & Sykes, B. D. (1992) *Biochemistry 31*, 8790−8798.

Vashishtha, A., & Fischetti, V. A. (1993) *J. Immunol. 150*, 4693−4701.

Wagner, B., Schmidt, K.-H., Wagner, M., & Köhler, W. (1986) *Zbl. Bakt. 261*, 432−446.

Whitnack, E., Dale, J. B., & Beachey, E. H. (1984) *J. Exp. Med. 159*, 1201−1212.

Zhou, N. E., Kay, C. M., & Hodges, R. S. (1992) *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.

BI962991S