Structure and Stability of Histone HMf from the Hyperthermophilic Archaeon *Methanothermus fervidus*†

Rowan A. Grayling,‡ Wayne J. Becktel,§ and John N. Reeve*,‡

Departments of Microbiology and Biochemistry, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210

Received November 21, 1994; Revised Manuscript Received February 16, 1995⊗

ABSTRACT: The secondary and quaternary structures and stabilities of recombinant (r) forms of the HMfA and HMfB histones from *Methanothermus fervidus* have been investigated by CD spectroscopy and formaldehyde-mediated protein−protein cross-linking. Both proteins were shown to be dimers in solutions containing 5−1300 mM KCl, at pH 6−10 and 25−83 °C, and specifically in 1 M KCl, at pH 7.5 and 83 °C, conditions which approximate those *in vivo* in *M. fervidus* cells. Heat treatment of a mixture of rHMfA and rHMfB homodimers resulted in the formation of rHMfA·rHMfB heterodimers, as demonstrated by two-dimensional PAGE. Heterodimer formation did not result in a CD-detectable conformational change from the homodimer states, indicating that homogeneous $(rHMfA)_2$ and $(rHMfB)_2$ preparations may be considered as structural models of heterodimers. At pH 2, both rHMfA and rHMfB were denatured under low-salt (<0.2 M KCl) conditions, and their conformations were stabilized in a cooperative manner by increasing KCl concentration, with cooperativity constants for KCl uptake of 2.7 and 3.1, respectively. The α-helical conformations of rHMfA and rHMfB were salt-dependent, at both pH 2 and pH 7.5, with maximal helicities in 1 M KCl of 84% and 63% at pH 2, and 72% and 65% at pH 7.5, respectively. The data obtained indicate that the structures of HMfA and HMfB, in 100-200 mM KCl at pH 7.5 and 25 °C, are likely to be very similar to their *in vivo* structures, even though these conditions are far removed from those found *in vivo*. Correlation of engineered primary sequence changes with their effects on the structures and stabilities of rHMfA and rHMfB established here should therefore be relevant to the *in vivo* situation.

Preparations of histone HMf, isolated from the hyperthermophilic archaeon (formerly archaebacterium) *Methanothermus fervidus*, contain two polypeptides, HMfA (MW[1] 7.4 kDa) and HMfB (MW 7.7 kDa), that have amino acid sequences that are 84% identical (Sandman et al., 1990; Tabassum et al., 1992). These polypeptides form a mixture of $(HMfA)_2$, $(HMfB)_2$, and predicted HMfA·HMfB dimers *in vivo*, in monomer ratios that are growth-phase-dependent (Sandman et al., 1994a). Closely related proteins have also been identified in mesophilic methanogens and in nonmethanogenic *Archaea* (Sandman et al., 1994b; Darcy et al., 1995), all of which, based on their amino acid sequences and predicted secondary structures, appear to share a common ancestor with the H2A, H2B, H3, and H4 histones that form the core structure of the eukaryal nucleosome (Grayling et al., 1994). These archaeal proteins appear to be functionally homologous to the histones, as they bind and compact DNA molecules into nucleosome-like structures (Sandman et al., 1990), and therefore may provide a simplified system to study histone−DNA interactions and nucleosome assembly. Furthermore, because the HMf proteins are also very small and are extremely stable, they are also very attractive as model proteins for studies of intrinsic protein stability.

The genes *hmfA* and *hmfB* that encode HMfA and HMfB, respectively, have been overexpressed in *Escherichia coli*, resulting in homogeneous preparations of recombinant HMfA (rHMfA) and recombinant HMfB (rHMfB). These recombinant polypeptides are identical to the native HMfA and HMfB polypeptides synthesized in *M. fervidus*, based on SDS−PAGE, reverse-phase HPLC, and N-terminal amino acid sequencing (Sandman et al., 1995). Here we report the structures and stabilities of rHMfA and rHMfB, determined over a wide range of salt and pH conditions, by CD spectroscopy, formaldehyde-mediated protein−protein cross-linking, and one- and two-dimensional gel electrophoresis. The results obtained establish the basis for a comprehensive dissection of the structure, function, and stability relationships of these proteins, using a site-directed mutagenesis approach.

MATERIALS AND METHODS

Reagents and Proteins. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Preparations of rHMfA and rHMfB were made as previously reported (Sandman et al., 1994a, 1995) and contained >98% rHMfA or rHMfB, as determined by silver-staining following SDS−PAGE, and by reverse-phase HPLC. Amino acid microsequencing demonstrated that >95% of the recombinant protein

† This work was supported by U. S. Office of Naval Research Grant N00014-92-J-1932 and The Ohio State University. R.A.G. was supported in part by a Monsanto Biotechnology Fellowship.

* To whom correspondence should be addressed.

‡ Department of Microbiology.

§ Department of Biochemistry.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1995.

[1] Abbreviations: CD, circular dichroism; MW, molecular mass; cDPG, cyclic 2,3-diphosphoglycerate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; tricine, *N*-[tris(hydroxymethyl)methyl]glycine; PAGE, polyacrylamide gel electrophoresis; % T, percent acrylamide plus *N*,*N*′-methylenebis(acrylamide) monomer (total monomer %); % C, percent *N*,*N*′-methylenebis(acrylamide) (cross-linker %).

0006-2960/95/0434-8441$09.00/0

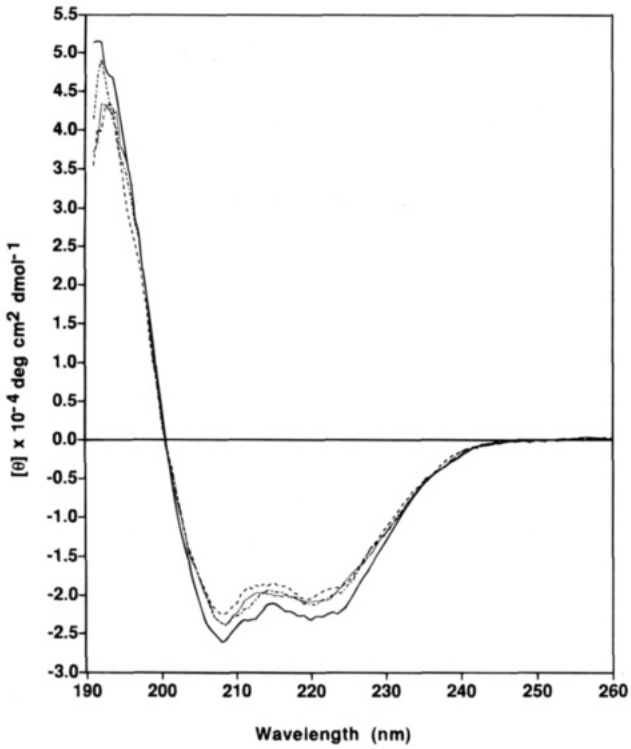

FIGURE 1: CD spectra of mixtures of $(rHMfA)_2$ and $(rHMfB)_2$ dimers. Spectra of protein samples (35 μM) in 100 mM KCl, 10 mM Tris-HCl, pH 7.5, were measured in a cylindrical cell with 0.1 mm path length. The spectra are $(rHMfA)_2$ (—), $(rHMfB)_2$ (- - -), an equimolar mixture of $(rHMfA)_2$ and $(rHMfB)_2$ (— • —), and an identical, but heat-treated, equimolar mixture of $(rHMfA)_2$ and $(rHMfB)_2$ (▪▪▪). The spectra shown were generated from raw data which were smoothed using a 5-point sliding Fourier filter.

Table 1: Physical Properties of rHMfA and rHMfB

protein	$[\theta]_{222,pH\ 2}$[a,b]	$[\theta]_{222,pH\ 7.5}$[a,b]	% helix, pH 2[c]	% helix, pH 7.5[c]
rHMfA[d]	−7900	−20 400	12 (31)	62 (70)
rHMfA[e]	−26 100	−23 100	84 (88)	72 (78)
rHMfB[d]	−5800	−18 700	3 (24)	55 (65)
rHMfB[e]	−20 800	−21 300	63 (71)	65 (73)

[a] The secondary structure of the rHMfB polypeptide is known from NMR data to consist of only α-helical regions and segments of disordered structure (M. Starich, K. M. Sandman, M. F. Summers, and J. N. Reeve, unpublished results). The values of molar ellipticity used to calculate helical content were therefore only taken at 222 nm, and are indicated in units of deg cm^2 dmol^{-1}. [b] Circular dichroism values used were taken from the same data sets used to generate Figure 4B,C. [c] Calculated on the basis of 100% α-helix = −30 000 (Chen et al., 1974) and 100% coil = −5 000 deg cm^2 dmol^{-1} (Becktel & Schellman, 1987). The values listed in parentheses indicate the percent helix calculated using +2 000 deg cm^2 dmol^{-1} as an alternative 100% coil value (Becktel & Schellman, 1987). [d] Proteins examined at 5 mM KCl. [e] Proteins examined at 1 M KCl.

in these preparations had the same N-terminal sequence as the respective native HMfA and HMfB proteins. The rHMfA and rHMfB polypeptides do not contain UV-absorbing residues suitable for use in protein quantitation. Protein concentrations were therefore determined in triplicate by quantitative amino acid analysis, using a Waters Picotag amino acid analysis system (Millipore Corp., Milford, MA). All protein concentrations stated refer to the concentration of rHMfA or rHMfB monomers. The amino acid analyses also indicated that the composition of each recombinant protein was identical to the corresponding native protein

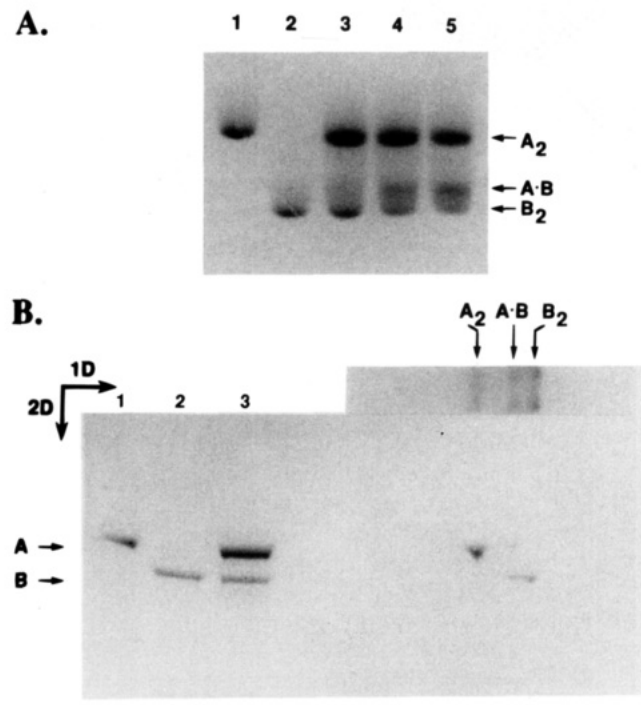

FIGURE 2: PAGE analysis of heat-treated $(rHMfA)_2$ and $(rHMfB)_2$ dimers and kinetics of heterodimer formation. (A) Single-phase, nondenaturing PAGE showing (lane 1) rHMfA homodimers (A_2, 4.5 μg), (lane 2) rHMfB homodimers (B_2, 4.5 μg), and lanes 3, 4, and 5) equimolar mixtures of rHMfA and rHMfB homodimers (9 μg), incubated for 45 min at 25 °C, for 3 h at 25 °C, and heat-treated at 95 °C (see text). The band predicted to contain rHMfA·rHMfB heterodimers is indicated (A·B). (B) Two-dimensional PAGE. The dimers separated in the first dimension (1D), by biphasic, nondentaturing PAGE, were resolved in the second dimension (2D) by denaturing acetic acid−urea-PAGE in the presence of 0.8% Triton X-100. The dimers are identified in the first dimension as in panel A. Lanes 1, 2, and 3 contained the same samples as described for lanes 1, 2, and 3 in panel A, and the denatured, monomeric rHMfA (A) and rHMfB (B) polypeptides are indicated. The different staining intensities of rHMfA and rHMfB result from differences in binding Coomassie Brilliant Blue R-250 in the particular PAGE system. That equimolar quantities of rHMfA and rHMfB were used was determined by quantitative amino acid analysis in triplicate.

obtained from *M. fervidus*. The recombinant proteins (10−20 mg/mL) were stored at −20 °C in 1 M KCl, 25 mM Tris-HCl (pH 7.5), and diluted as appropriate for each experiment. Mixtures containing (rHMfA·rHMfB) heterodimers were prepared by incubating homogeneous preparations of rHMfA and rHMfB in 100 mM KCl, 10 mM Tris-HCl (pH 7.5), in equimolar amounts at 95 °C, followed by slow-cooling to room temperature, as previously described (Sandman et al., 1994a). These (heat-treated) mixtures were used for both electrophoresis and CD studies.

Electrophoresis Techniques. (A) One Dimension. The HMf proteins migrate with the ion front in standard SDS−PAGE systems. Therefore, a tricine−SDS−PAGE system with a 16.5% T, 3% C acrylamide resolving gel and a 10% T, 3% C spacer gel, which separates such small proteins from the ion front, was used to resolve the products of the cross-linking reactions (Schägger & von Jagow, 1987).

Two different nondenaturing (native) electrophoresis systems were used to separate the dimers in HMf preparations on the basis of differences in their net positive charges. A continuous, single-phase system was used to resolve the components of the heat-treated mixtures of rHMfA and

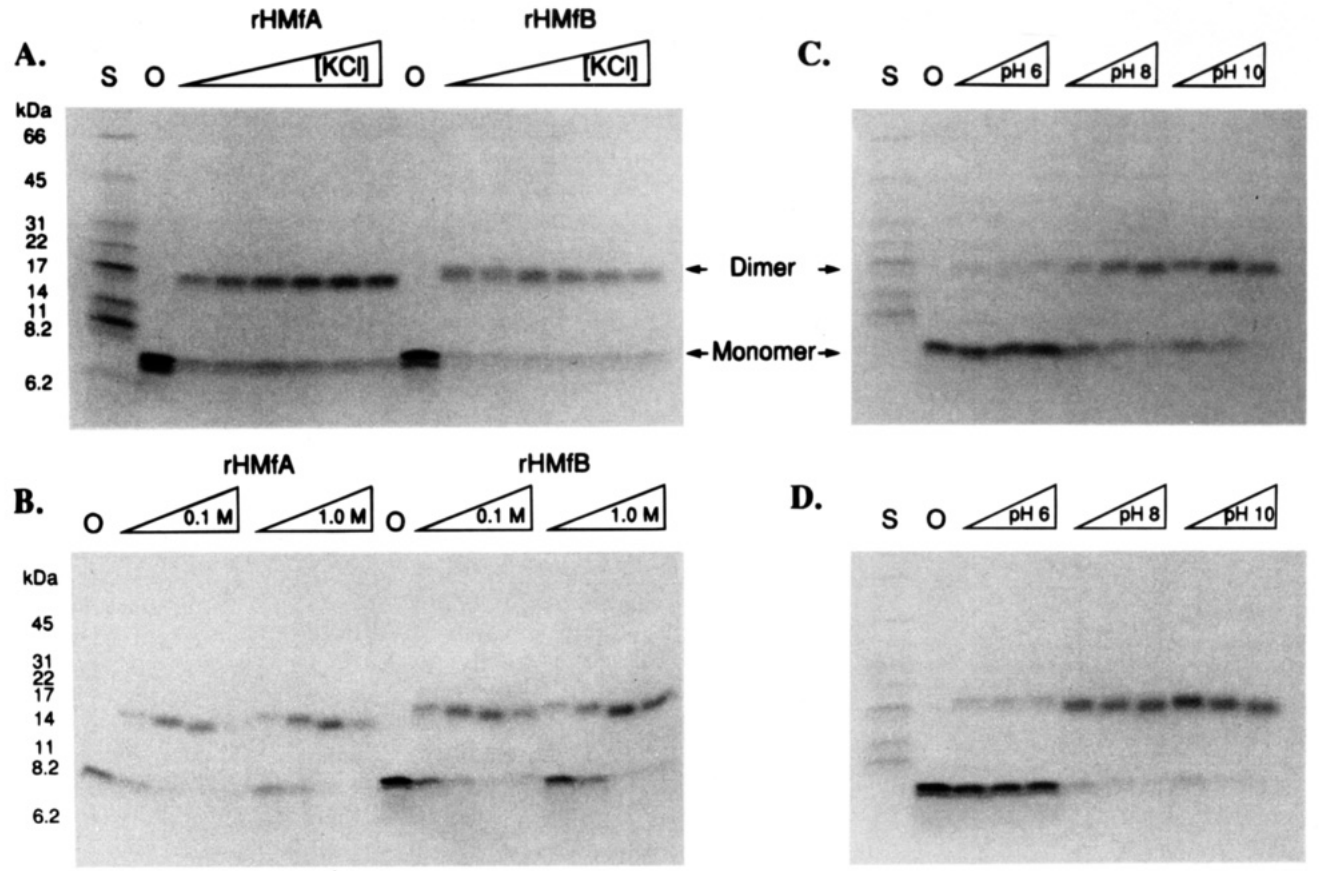

FIGURE 3: Tricine−SDS−PAGE of the products of formaldehyde cross-linking. Preparations of rHMfA and rHMfB (1.5 μg samples) were cross-linked. (A) In the presence of 0, 50, 100, 500, 1000, and 1500 mM KCl at pH 7.5. (B) In the presence of 0.1 or 1 M KCl, at pH 7.5, at 25, 37, 65, 83 °C. (C and D) Time course of cross-linking of rHMfA and rHMfB, respectively, at pH 6, 8, and 10, at 25 °C, in 100 mM KCl for 20, 40, or 80 min. Symbols indicate: O, no cross-linker added (100 mM KCl, pH 7.5); S, protein size standards. The positions of monomer and dimer forms are indicated.

rHMfB that were analyzed by CD spectroscopy. This system, which has been described previously in detail (Sandman et al., 1994a), uses a histidine−MES buffer at pH 6.1, and has no detectable effect on the kinetics of rHMfA·rHMfB heterodimer formation, in part due to the absence of a stacking gel. A discontinuous, two-phase system was developed from first principles, as discussed by Williams and Reisfeld (1964) and Richards et al. (1965), and employed a 4% T, 3% C acrylamide stacking gel, and a 16.5% T, 3% C resolving gel. This discontinuous system gave better, more reproducible resolution of the different HMf dimer populations than the continuous system. The stacking gel contained 30 mM PIPES, 10% (v/v) glycerol, adjusted to pH 7.4 with KOH, and the resolving gel contained 70 mM PIPES, 10% (v/v) glycerol, adjusted to pH 6.5 with KOH. The electrode buffer was 84 mM histidine, 37 mM PIPES, (pH 6.1). Protein samples were mixed with an equal volume of 30 mM PIPES, 20% (v/v) glycerol, 0.005% (w/v) Pyronin Y (adjusted to pH 7.4 with KOH), before being loaded on the gel. Electrophoresis with the discontinuous system was carried out at 10−12 V/cm, for 4−6 h.

(B) Two Dimensions. Electrophoresis in a second dimension was used to separate and identify the components of the dimer bands that formed during nondenaturing electrophoresis using the discontinuous system. A denaturing acetic acid−urea−PAGE system that incorporated 0.8% (v/v) Triton X-100 (Smith, 1984) was used in the second dimension. Proteins resolved in the second dimension were quantified by densitometry using known amounts of rHMfA and rHMfB as standards.

Protein−Protein Cross-Linking. Formaldehyde and dimethyl suberimidate cross-linking of rHMfA and rHMfB molecules in solution gave identical results (R.A.G., unpublished experiments). The results documented here were obtained by the addition of 1 μL of 400 mM formaldehyde to 9 μL of a solution containing rHMfA or rHMfB (1.5 μg), KCl (diluted as appropriate from a 3.5 M stock solution), and 25 mM of the appropriate buffer (diluted from a 100 mM stock solution). The buffers used were potassium phosphate, pH 6; potassium phosphate, pH 7.5; triethanolamine hydrochloride, pH 8; and sodium borate, pH 10. The reaction mixtures were incubated at 25 °C for 1 h (unless otherwise indicated in the legend to Figure 2), and then quenched by addition of 1 μL of 0.5 M ammonium bicarbonate, and continued incubation for 15 min at 25 °C. The reaction mixtures were then dialyzed against 100 mM NaCl, 25 mM Tris-HCl (pH 7.5), mixed with an equal volume of sample buffer [8% (w/v) SDS, 20% (v/v) glycerol, 100 mM Tris-HCl, 4% (v/v) 2-mercaptoethanol, and 0.01% (w/v) bromophenol blue (pH 6.8)], and loaded onto tricine−SDS gels. Cross-linking was not complete, even at pH 10, and therefore some rHMfA and rHMfB monomers were always detected following electrophoresis.

Circular Dichroism (CD) Spectroscopy. CD spectra of 35 μM or 5 μM protein solutions, in buffers that had been passed through 0.2 μm filters, were measured at ambient temperature (25 °C) using a Spex CD6 spectropolarimeter.

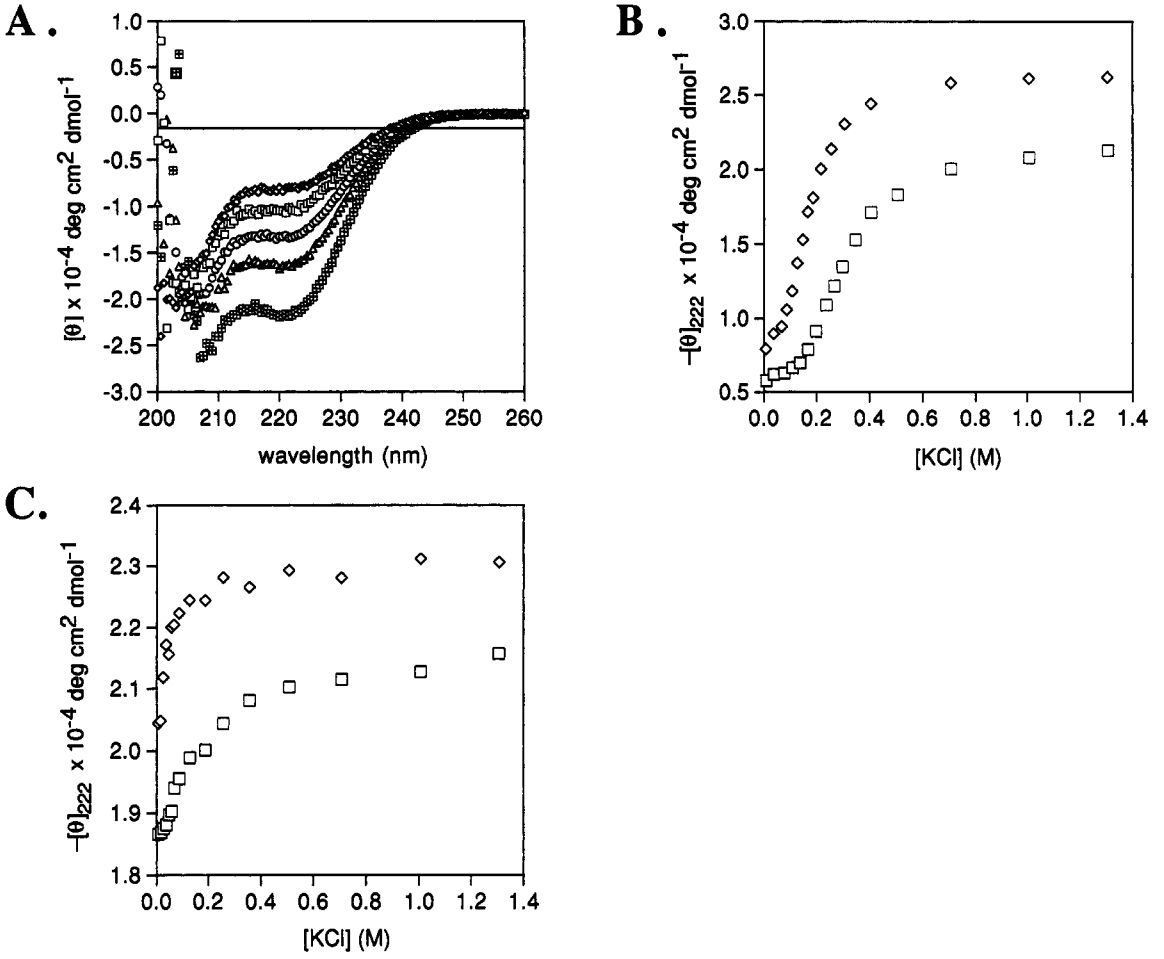

FIGURE 4: Salt titrations of rHMfA and rHMfB at pH 2 and 7.5. (A) A subset of the CD spectra for rHMfA at pH 2, measured in 0.006 M KCl (◇), 0.086 M KCl (□), 0.126 M KCl (○), 0.166 M KCl (△), and 0.406 M KCl (open square with + inscribed). (B and C) Circular dichroism measured at 222 nm as a function of KCl concentration, at pH 2 and 7.5, respectively. The data were collected using protein concentrations of 5 μM, in a 1 cm path length rectangular cell for rHMfA (◇), and for rHMfB (□).

For the heat-treatment experiment, proteins were examined in 100 mM KCl, 10 mM Tris-HCl (pH 7.5). For the salt titrations, protein stock solutions were diluted directly into filtered 0.01 N HCl (pH 2) or 10 mM Tris-HCl (pH 7.5). For each titration, 2.3 mL of the appropriate protein solution was dispensed into the cuvette, and 3.5 M KCl was serially added to give the desired final KCl concentration. The contents of the cell were mixed and incubated at ambient temperature for 10 min after each salt addition, to allow equilibrium to be attained before measurement. The pH variation over the salt range of the titrations was <0.1 unit, and overall errors for these data varied from $<\pm 1\%$ for KCl concentrations ≤ 0.2 M to $<\pm 0.3\%$ for KCl concentrations ≥ 0.2 M. Data were acquired at 0.5 nm intervals, and 4−16 scans were accumulated for each spectrum. Data from the heat-treatment experiment (Figure 1) were smoothed with a sliding Fourier filter. Errors for the unsmoothed data points in the region 218−230 nm were $<\pm 3\%$. For all measurements, instrument base-line drift was < 0.1 mdeg per day and base-line deviation from 0 mdeg in nonabsorbing spectral regions was $<\pm 0.5$ mdeg. Assymptotes to sigmoidal curves for the calculation of ion uptake at pH 2 were determined by nonlinear regression. All circular dichroism values were calculated as molar ellipticities ([θ], in units of degrees centimeter squared per decimole for dilution-corrected data using molecular masses of 7369 and 7667 Da for rHMfA and rHMfB monomers, respectively.

RESULTS

Secondary Structures of rHMfA and rHMfB. The CD spectra of rHMfA and rHMfB homodimers in 100 mM KCl at pH 7.5 are shown in Figure 1. They are very similar, but are not identical, and no detectable changes in either spectrum were observed using protein concentrations ranging from 1 to 50 μM (data not shown). On the basis of the dichroism at 222 nm, $(rHMfA)_2$ molecules have slightly more helical character than $(rHMfB)_2$ molecules (Table 1).

At pH 6.5, $(rHMfA)_2$, rHMfA·rHMfB, and $(rHMfB)_2$ dimers are predicted to have net charges of +3.1, +5.1, and +7.1, respectively (Devereux et al., 1984). When mixtures of $(rHMfA)_2$ and $(rHMfB)_2$, that had been heat-treated at 95 °C, were analyzed using the single-phase, nondenaturing PAGE system at pH 6.5, three bands were obtained that had mobilities consistent with the presence of $(rHMfA)_2$, rHMfA·rHMfB, and $(rHMfB)_2$ (Figure 2A). This mixture resembled the mixture of dimers present in native HMf preparations (Sandman et al., 1994a), and therefore the composition of the band predicted to contain rHMfA·rHMfB heterodimers was established conclusively by two-dimensional PAGE. Components of the dimers separated by nondenaturing PAGE in the first dimension were resolved as denatured monomers in the second dimension, which demonstrated that the band previously assumed to contain rHMfA·rHMfB heterodimers (Figure 2A) did indeed contain

equal amounts of the rHMfA and rHMfB monomers (Figure 2B). Both heat-treated and equivalent, non-heat-treated mixtures of $(rHMfA)_2$ and $(rHMfB)_2$ were examined by CD spectroscopy (Figure 1). Identical CD spectra were obtained for both mixtures, and these lie between the spectra obtained for the homodimers alone. The single-phase, nondenaturing PAGE system was therefore used to confirm that heterodimer formation had occurred in the heat-treated mixture, and that there was negligible heterodimer formation in the nonheated mixture during the time needed to acquire its CD spectrum (Figure 2A).

Quaternary Structure of rHMfA and rHMfB. Native HMf preparations, in 100 mM potassium phosphate (pH 6.9), were shown previously by low-resolution, size-exclusion chromatography to consist of polypeptide dimers (Krzycki et al., 1990). Formaldehyde-mediated covalent cross-linking (Ji, 1983) combined with analysis by tricine−SDS−PAGE has now been used to determine the oligomeric state of the rHMfA and rHMfB proteins over a wide range of salt, pH, and temperature conditions, many of which were incompatible with other cross-linking reagents or chromatographic methods. For both proteins, cross-linking generated products with the electrophoretic mobility expected for dimers (MW ~15 kDa), under all solution conditions tested (Figure 3). The variations observed in the extent of cross-linking were consistent with the dependence of the cross-linking reaction on the proportion of lysine residues expected to be unprotonated at the reaction pH (Figure 3C,D), and with the reaction temperature (Figure 3B). Dimers were the only cross-linked products observed at protein concentrations ranging from 1 to 50 μM, even with the addition of excess formaldehyde, or prolonged incubation of reaction mixtures (data not shown).

M. fervidus grows optimally at 83 °C, and has an internal potassium concentration of ~1 M with the major counterion being cyclic 2,3-diphosphoglycerate (cDPG) (Hensel & König, 1988). K_3cDPG is not commercially available, and is difficult to purify in preparative amounts from *M. fervidus*. KCl was therefore substituted to generate conditions that approximated the *in vivo* situation [1 M K^+, 83 °C (pH 7.5)]. Under these conditions (Figure 3B), and in fact at all KCl concentrations tested, both rHMfA and rHMfB were polypeptide dimers in solution (Figure 3A).

Salt Titrations of rHMfA and rHMfB. The stabilities of the rHMfA and rHMfB secondary structures were determined by CD spectroscopy, as a function of KCl concentration, at pH 2 and pH 7.5. A subset of the CD spectra obtained for rHMfA at pH 2 is shown in Figure 4A, and very similar spectra were obtained for rHMfB (data not shown). At pH 2, rHMfA and rHMfB exhibited large changes in dichroism at 222 nm, showing cooperative transitions with increasing KCl concentration. Complete denaturation was achieved at pH 2, but only at KCl concentrations <0.1 M (Figure 4B).

At pH 7.5, the changes in the 222 nm dichroism of rHMfA and rHMfB proteins with increasing KCl concentration were greatly reduced, but the transitions observed were more cooperative and occurred at lower salt concentrations than those observed at pH 2. Although the helicity of both proteins increased with increasing KCl concentration at pH 7.5, at salt-saturation (>0.6 M KCl) the dichroism of rHMfA at 222 nm reached only −23 100 deg cm^2 dmol^{-1}, a value

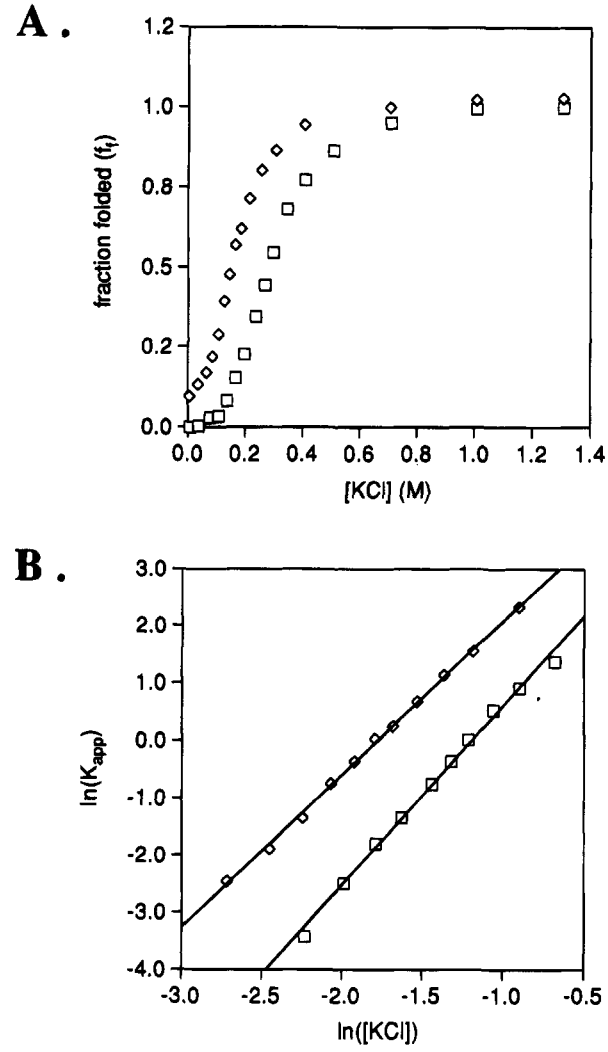

FIGURE 5: Ion uptake calculations for the folding reactions of rHMfA and rHMfB at pH 2. (A) Fraction of protein folding (f_f) versus KCl concentration. (B) Least-squares fit of the natural logarithm of the apparent equilibrium constants for conversion between the low- and high-salt (unfolded and folded) forms of the proteins versus the natural logarithm of KCl concentration. Data in both plots correspond to rHMfA (◇) and rHMfB (□). The slopes calculated were 2.7 and 3.1 for rHMfA and rHMfB, respectively.

13% less than the dichroism of rHMfA measured at saturating salt conditions at pH 2 (−26 100 deg cm^2 dmol^{-1}). Measured dichroism values and estimated helicities for the salt titrations are summarized in Table 1. At pH 2, rHMfA showed a 230% increase in dichroism at 222 nm over the salt range from 5 mM to 1.3 M KCl, but only a 13% increase was observed at pH 7.5. For rHMfB, the increases were 260% and 14%, respectively.

Ion Uptake by rHMfA and rHMfB at Acidic pH. The effect of KCl concentration on the extent of folding of rHMfA and rHMfB can be estimated from the slope of a Hill-type plot of $\ln(K_{app})$ versus $\ln(c)$, where c is the KCl concentration and K_{app} is the apparent equilibrium constant for renaturation. The value of the slope obtained is a measure of the cooperativity of ion uptake during the folding reaction. Plots of the fractional unfolding of rHMfA and rHMfB versus the KCl concentration are sigmoidal, suggesting a single transition for both proteins at pH 2 (Figure 5A). If a unimolecular, two-state renaturation reaction is assumed for

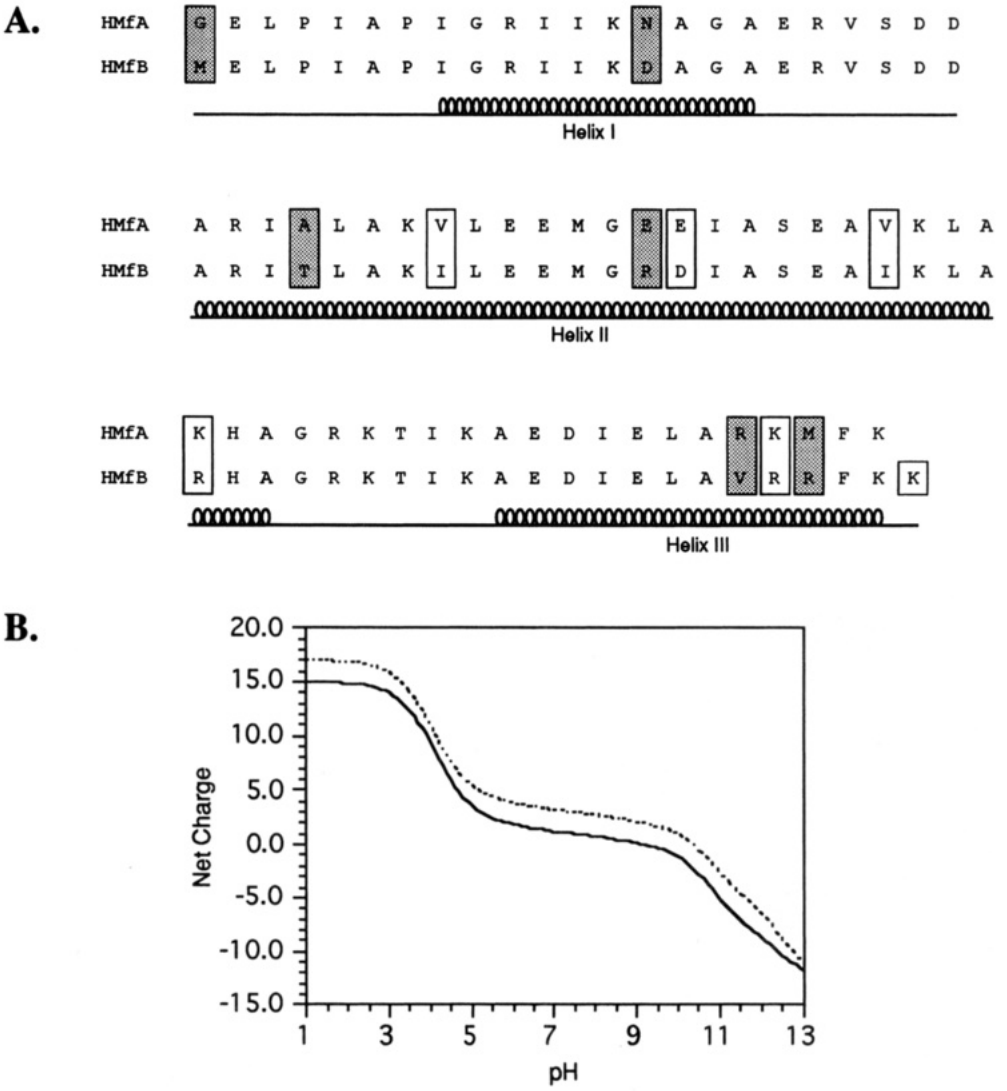

FIGURE 6: Predicted differences in the stabilities of HMfA and HMfB. (A) Alignment of the amino acid sequences of HMfA and HMfB. Residues that differ between the two sequences are boxed, where shaded boxes indicate nonconservative differences. Three regions of the proteins predicted to have α-helical secondary structures are indicated by the coiled lines, and the remaining regions are predicted to be unstructured (Grayling et al., 1994). (B) pH titration curves predicted for the HMf proteins. The algorithm used is incorporated in the ISOELECTRIC program (Devereux et al., 1984), and assumes that each amino acid titrates independently with pK_a values corresponding to those of the free amino acids. Solid and dashed lines indicate the formal net charges predicted for rHMfA and rHMfB, respectively, over the pH range 1−13.

the proteins at pH 2, the apparent equilibrium constants can be calculated as

$$K_{app} = \frac{f_f}{1 - f_f}$$

and

$$f_f = \frac{[\theta] - [\theta]_u}{[\theta]_f - [\theta]_u}$$

where $[\theta]_u$ is the dichroism of the unfolded (ion unbound) form, $[\theta]_f$ is the dichroism of the folded (binding site saturated) form, and f_f is the fraction of the folded form. A plot of $\ln(K_{app})$ vs $\ln(c)$ yields linear fits with slopes of 2.7 and 3.1 for rHMfA and rHMfB, respectively (Figure 5B), indicating that these are highly cooperative transitions.

DISCUSSION

We have determined the secondary and quaternary structures and stabilities of rHMfA and rHMfB over a wide range of conditions. Formaldehyde cross-linking has demonstrated that these proteins are polypeptide dimers in solution over very wide ranges of salt, pH, and temperature conditions, including the 1 M K^+, 83 °C, pH 7.5 condition that was used to approximate the *in vivo* environment. The CD data demonstrated that rHMfA and rHMfB also have very stable secondary structures, provided that sufficient salt is present. At pH 7.5, there is little change in the helical content of either of these proteins above ~100 mM KCl (Figure 4C), indicating that experiments carried out *in vitro* at neutral pH in the presence of ≥100 mM KCl should generate results that are relevant to the HMf structure *in vivo*.

Heating mixtures of $(rHMfA)_2$ and $(rHMfB)_2$ generated rHMfA·rHMfB heterodimers and resulted in dimer mixtures similar to those found in native HMf preparations from *M. fervidus* (Sandman et al., 1994a). These heat-treated mixtures and identical, non-heat-treated mixtures had identical CD spectra, and exhibited dichroism values between those of $(rHMfA)_2$ and $(rHMfB)_2$. Heterodimer formation therefore does not result in a conformational change that differs

detectably from the homodimeric states, indicating that results of studies of rHMfA and rHMfB homodimers may be extrapolated legitimately to infer structural properties of the heterodimers. This is important as heterodimers are present *in vivo*, but without procedures to generate homogeneous preparations of heterodimers, their structure cannot be investigated directly.

Many of the primary sequence differences between rHMfA and rHMfB are conservative amino acid substitutions that lie within regions of the proteins that are predicted to form amphipathic α-helices (Figure 6A) (Grayling et al., 1994). There are, however, four nonconservative substitutions (N14D, E37R, R64V, and M66R) in these regions that should cause a net charge difference between rHMfA and rHMfB. Differences in electrostatic interactions within these proteins are therefore likely to be responsible for the differences in their salt-dependent stabilities at low pH (Figure 4B,C).

Estimates of the role of such electrostatic interactions on the stabilities of HMfA and HMfB can be made by examining pH titration curves, predicted by making the assumption that each titrable group within these proteins titrates independently, and with the same pK_a as the corresponding free amino acid. The curves obtained indicate that the net charges on HMfA and HMfB should remain parallel, as a function of pH, with HMfB maintaining approximately a +2 greater formal net charge over the entire pH range (Figure 6B). Electrostatic interactions should therefore be greater for HMfB than for HMfA at all pH values, but particularly at acidic pHs, where these proteins have high positive charges. Furthermore, added salt should stabilize both proteins through electrostatic charge shielding, and this effect should be greater for HMfB than for HMfA. These predictions are consistent with the salt titration data acquired at pH 2. Complete denaturation of rHMfB, but only partial denaturation of rHMfA, was observed at pH 2 and <0.1 M KCl concentrations (Figure 4B), conditions where ion-dependent shielding of ionized groups should be least effective. Also, both proteins were stabilized at pH 2 by increasing KCl concentration, and KCl uptake was more cooperative for rHMfB than for rHMfA, as indicated by cooperativity constants of 3.1 and 2.7, respectively. This degree of cooperativity could result from site-specific ion binding (most likely by chloride ions), but there are, as yet, no experimental data that address this parameter. It is not clear, however, why at the higher salt concentrations the helicity of HMfA at pH 2 (84%) was so different from that at pH 7.5 (72%). In addition to electrostatic interactions, other factors associated with protein stabilization, such as intrahelical salt bridges (Marqusee & Baldwin, 1987), high alanine content (Menéndez-Arias & Argos, 1989), and increased hydrophobic interaction, probably also contribute to the stability profiles of these proteins at low pH.

The large changes observed in helicity at pH 2, and the parallel nature of the pH titration curves (Figure 6B), indicate that studies of the HMf proteins using low salt conditions, away from neutral pH, will not generate results meaningful to the *in vivo* situation. Nevertheless, the contributions of individual amino acid residues to the stabilities and helical contents of the HMf proteins can now be examined using the conditions established here. Stepwise conversion of rHMfA into rHMfB, by site-directed mutagenesis, should demonstrate which residues, individually or in combination, confer increased stability and helicity on rHMfA at pH 2, and to what extent electrostatic interactions are involved at all pH values.

The HMf proteins are also very resistant to heat inactivation (Darcy et al., 1995), and we have yet to establish conditions that result in complete denaturation of their secondary structures, using only heat or heat combined with urea or guanidine. Recently, HFo preparations were isolated from *Methanobacterium formicicum*, a mesophile that grows optimally at 37 °C (Darcy et al., 1995). These preparations contain three polypeptides that have primary sequences that are ~80% identical to the HMf polypeptides. The DNA binding activity of these HFo preparations is, however, much less thermostable ($t_{1/2}$ at 95 °C of ~1.5 h) than that of HMf preparations ($t_{1/2}$ at 95 °C of >5 h) (Darcy et al., 1995). Mesophiles probably evolved from thermophiles (Pace, 1991; Adams, 1993), and therefore many of the structural features that confer high intrinsic heat resistance and salt-dependent stability on the thermophilic[2] HMf proteins are likely to have been retained in the mesophilic HFo proteins. Some of the few differences that do exist in the primary sequences of the HFo and HMf proteins (Darcy et al., 1995) must however be responsible for the reduced thermal stability of the HFo proteins. These can now be identified by using site-directed mutagenesis to generate protein variants, and assaying the thermal stabilities of their DNA binding activities. The effects of these changes on the stabilities and secondary structures of HMfA and HMfB can also be determined, by CD spectroscopy studies of the protein variants using the conditions established in this study.

[2] The terms *mesophilic* and *thermophilic* are used to identify proteins isolated from mesophilic and thermophilic organisms, respectively, following the convention used by Adams (1993).

ACKNOWLEDGMENT

We thank K. Sandman for providing the *E. coli* clones used to produce rHMA and rHMB, and D. Ordaz and the OSU Fermentation Facility for assistance in recombinant protein production.

REFERENCES

Adams, M. W. W. (1993) *Annu. Rev. Microbiol. 47*, 627−658.
Becktel, W. J., & Schellman, J. A. (1987) *Biopolymers 26*, 1859−1877.
Chen, Y.-H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350−3359.
Darcy, T. J., Sandman, K., & Reeve, J. N. (1995) *J. Bacteriol. 177*, 858−860.
Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res. 12*, 387−395.
Grayling, R. A., Sandman, K., & Reeve, J. N. (1994) *System. Appl. Microbiol. 16*, 582−590.
Hensel, R., & König, H. (1988) *FEMS Microbiol. Lett. 49*, 75−79.
Ji, T. H. (1983) *Methods Enzymol. 91*, 580−609.
Krzycki, J. A., Sandman, K., & Reeve, J. N. (1990) in *Proceedings of the 6th International Symposium on the Genetics of Industrial Microorganisms* (Heslot, H., Davies, J., Florent, J., Bobichan, L., Durand, G., & Penasse, L., Eds.) pp 603−610, Societé Français de Microbiologie, Strasbourg, France.
Marqusee, S., & Baldwin, R. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8898−8902.
Menéndez-Arias, L., & Argos, P. (1989) *J. Mol. Biol. 206*, 397−406.

Pace, N. (1991) *Cell 65*, 531−533.
Richards, E. G., Coll, J. A., & Gratzer, W. B. (1965) *Anal. Biochem. 12*, 452−471.
Sandman, K., Krzycki, J. A., Dobrinski, B., Lurz, R., & Reeve, J. N. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5788−5791.
Sandman, K., Grayling, R. A., Dobrinski, B., Lurz, R., & Reeve, J. N. (1994a) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12624−12628.
Sandman, K., Perler, F. B., & Reeve, J. N. (1994b) *Gene 150*, 207−208.
Sandman, K., Grayling, R. A., & Reeve, J. N. (1995) *Bio/Technology 13*, 504−506.
Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
Smith, B. J. (1984) in *Methods in Molecular Biology: Proteins* (Walker, J. M., Ed.) pp 63−73, Humana Press, Clifton, NJ.
Tabassum, R., Sandman, K. M., & Reeve, J. N. (1992) *J. Bacteriol. 174*, 7890−7895.
Williams, D. E., & Reisfeld, R. A. (1964) *Ann. N. Y. Acad. Sci. 121*, 373−381.

BI942690N