High Level Expression and Dimer Characterization of the S100 EF-hand Proteins, Migration Inhibitory Factor-related Proteins 8 and 14*

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The phenotypical and functional heterogeneity of different macrophage subpopulations are defined by discrete changes in the expression of two S100 calcium-binding proteins, migration inhibitory factor-related proteins (MRPs) 8 and 14. To further our understanding of MRP8 and MRP14 in the developmental stages of inflammatory responses, overexpression of the MRPs was obtained through a combination of a T7-based expression vector and the *Escherichia coli* BL21 (DE3) cell line. An efficient, two-step chromatographic protocol was then developed for rapid, facile purification. Extensive biophysical characterization and chemical cross-linking experiments show that MRP8 and MRP14 form oligomers with a strong preference to associate as a heterodimer. Heteronuclear NMR experiments indicate that a specific well packed dimer is formed only in equimolar mixtures of the two proteins. Our results suggest that there is a unique complementarity in the interface of the MRP8/MRP14 complex that cannot be fully reproduced in the MRP8 and MRP14 homodimers.

Macrophages, which belong to the mononuclear phagocyte system, form a heterogeneous cell population with different developmental and functional stages. The specific pathophysiological situation dictates whether macrophages will perform cytotoxic, endocytic, or secretory functions. The phenotypical and functional heterogeneity of different macrophage subpopulations are defined by discrete changes in the expression of two S100 calcium-binding proteins, migration inhibitory factor-related proteins (MRPs) 8 and 14 (1–3). Granulocytes, monocytes/macrophages, neutrophils, and keratinocytes have been shown to express MRP8 and MRP14, suggesting that expression is tightly regulated during granulocyte differentiation (4–9). In addition, elevated serum levels of MRP8 and MRP14 have been found in patients suffering from a number of inflammatory disorders including cystic fibrosis, rheumatoid arthritis, and chronic bronchitis (10–13), suggesting possible cellular roles for these proteins. In the new S100 protein nomenclature, MRP8 and MRP14 are designated as S100A8 and S100A9, respectively (14). Other names for the MRP8/MRP14 complex include calprotectin (15), leukocyte-derived protein (L1) light and heavy chains (16), p8/p14 (17), calgranulin A/B (9), and the cystic fibrosis antigen (11, 18). Some evidence suggests that MRP14 alone is the cystic fibrosis antigen (19), and murine MRP8 has been referred to as the murine chemotactic cytokine (CP-10) (20). It has been suggested that MRP8 and MRP14 form a heterodimeric complex in a calcium-dependent manner (21). However, once this complex is formed, it is stable upon the addition of EDTA (22). It is not clear whether these proteins have independent biological functions or whether their function is strictly dependent on heterocomplex formation. Although the general consensus is that protein function is dependent on heterodimer formation, studies also support the possibility that these proteins may have individual functions. For example, MRP14 is shown to be expressed independently of MRP8 in acutely inflamed tissues (23–25).

Like a number of other S100 proteins (S100a, S100D, S100E, S100L, p11, CAPL, calycin/2A9, and psoracin), the genes encoding MRP8 and MRP14 have been located on chromosome 1q21 (11, 26, 27). Recently, it has been shown that the structure of the gene loci encoding S100 proteins is highly conserved (28). Although the primary protein sequences are similar, there is less similarity at the nucleotide level, with the greatest divergence in the untranslated regions. Since S100 proteins exhibit very specific patterns of expression, it has been suggested that these untranslated regions may be involved in directing the expression of specific S100 proteins in specific cell types (28). Analysis of the amino acid sequences of MRP8 and MRP14 indicate that each protein is composed of two EF-hand domains characteristic of the S100 calcium-binding protein family (27, 29, 30). S100 proteins are characterized by relatively low molecular masses, generally on the order of 10 kDa, and a unique consensus sequence in their calcium-binding sites making them a diverse, multifaceted family of proteins (31). They have been shown to play roles in cell cycle progression, regulation of intracellular phosphorylation events, and cytoskeletal membrane interactions, as well as extracellular events such as the stimulation of glial proliferation, prolactin secretion, and neuronal differentiation (30, 32).

MRP8 and MRP14 have molecular masses of 11 and 14 kDa and are composed of 93 and 114 amino acids, respectively. A number of possible functions for these proteins have been proposed including differentiation of myeloid lineage cells, regulation of intracellular calcium levels in neutrophils, inhibition of casein kinase I and II activity, and a zinc-mediated biostatic activity (19, 21, 33, 34). The extended carboxyl-terminal tail of MRP14 makes it the largest member of the S100 family, and it is believed that MRP14 represents the regulatory unit of this unique heterodimeric complex. Studies have shown that the...
Expression and Characterization of MRP8 and MRP14

penultimate amino acid of MRP14, Thr\textsuperscript{113}, is phosphorylated \textit{in vivo} (8, 35) and that the phosphorylation event triggers the translocation of this protein to the cytoskeleton (36). Possible functions for the tail of MRP14 may include neutrophil immobilization (37), regulation of cytoskeletal translocation of the MRP8/MRP14 complex (36), and inhibition of the onset of the intrinsic coagulation cascade (38). The only function assigned to MRP8 to date is the potent chemotactic activity assigned to linker region of murine MRP8 (20, 39–41).

To further our understanding of the roles MRP8 and MRP14 play in the developmental stages of inflammatory responses, we have cloned these two proteins and developed highly efficient expression and purification protocols. This has allowed for biophysical characterization by UV, CD, fluorescence, and heteronuclear NMR spectrosopies, as well as chemical cross-linking experiments. MRP8 and MRP14 are shown to be stable globular proteins that form dimers and preferentially associate as heterodimers.

**EXPERIMENTAL PROCEDURES**

**Construction of the Expression Vectors—**The MRP8 and MRP14 cDNAs were a generous gift of Professor Clemens Sorg (University of Münster, Germany). The primers MRP8–5′ (5′-GGA ATT CCA TAT GTT GAC GCA GCT GGA GAA-3′) and MRP8–3′ (5′-CGG GAT CCT GGC TTC CTT C-3′) were used to PCR amplify the human MRP8 gene from the pMRP-8-primers expression vector. A BamHI restriction site was engineered into the 5′-primers, and a BamHI restriction site was engineered into the 3′-primers for proper insertion of the gene into the pET1120 expression vector. Amplification was conducted in a total volume of 100 µl containing 10 mM KCl, 20 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM MgSO\textsubscript{4}, 1% Triton X-100, and 100 µM of each primer. A small amount of mineral oil was added to the top of the reaction mixture to inhibit evaporation. The reaction mixtures were heated to 95 °C for 5 min, followed by 25–30 cycles of 95, 60, and 75 °C each and a final extension at 75 °C for 2 min.

PCR products were purified using a Qiagen PCR purification kit (Qiagen, Inc., Chatsworth, CA). Full-length oligonucleotides were doubly digested with 20 units BamHI and 40 units NdeI for 2 h at 37 °C. The digested fragments were purified on a 1.3% agarose gel and stained with ethidium bromide. The bands containing the MRP8 and MRP14 genes were excised and purified using a Qiagen II gel extraction kit. The MRP8 and MRP14 genes were then cloned into the NdeI and BamHI sites of the pET1120 vector using standard methods (42). The pET vectors were transformed into competent E. coli DH5α cells. 2 µl of Novagen’s pET11 and pET20 vectors and ligating the large fragment of pET20 digestion with the small fragment of pET11 digestion. These plasmids have the β-lactamase gene and multiple cloning sites downstream of the efficient T7-polymerase promoter but are void of the lac repressor gene. Competent DH5α cells were transformed with the pET1120-MRP8wt and pET1120-MRP14wt vectors and used to produce plasmid stocks. Proper insertion and DNA sequences were confirmed by fluorescent thermal dye DNA sequencing methods (43).

**Mutagenesis—**To construct the MRP8C42S gene, the primers, MRP8C42S-5′ (5′-CTC GTC GCT GAG CAA TAT CCT C-3′) and MRP8–5′ were used to PCR amplify the 5′-half of the MRP8C42S gene, and the primers MRP8C42S-3′ (5′-TTG CCC TAT GAC ACC GGT TCT CCT CAG TAT ATC-3′) and MRP8–3′ were used to PCR amplify the 3′-half of the MRP8C42S gene. The two amplified half-fragments were purified on a 1.5% agarose gel and visualized using ethidium bromide. The bands containing the two fragments were excised and purified using a Qiagen II gel extraction kit (Qiagen). The two purified half-fragments were used as primers for each other and the full gene was made using the PCR methods described above. Since this did not result in the amplification of a full-length fragment, a small amount of the full-length mutant gene, which was used as a template to PCR amplify the full-length MRP8C42S gene using MRP8–5′ and MRP8–3′ as the amplification primers. To construct the MRP14CBS gene, the primers MRP14CBS-5′ (5′-GGA ATT CCA TAT GAC TAG TAA AAT GTC GCA GCT GG-3′) and MRP14–5′ were used with the wild-type template in one PCR step. Transformation and sequencing of mutants were performed as described for the wild-type proteins.

**Protein Expression—**Overexpression of the gene products was achieved in E. coli strain BL21(DE3). For unlabelled protein, cells were grown at 37 °C in (2× YT) media supplemented with ampicillin to a final concentration of 100 µg/ml. 5 ml of an overnight culture grown in the same media. Cells were allowed to grow another 18–24 h before harvesting. Since the pET1120 plasmid contains no lac repressor gene, protein expression was not tightly controlled, and the system was leaky enough to produce large amounts of protein in rich media without the need for induction.

For \textsuperscript{15}N-labeled protein, cells were grown at 37 °C in M9 minimal media supplemented with ampicillin to a final concentration of 100 µg/ml. 5 ml of an overnight culture grown in the same media. Cells were allowed to grow another 18–24 h before harvesting. Since the pET1120 plasmid contains no lac repressor gene, protein expression was not tightly controlled, and the system was leaky enough to produce large amounts of protein in rich media without the need for induction.

The MRP8 and MRP14 were isolated by preparative reverse-phase HPLC on a DeltaPak C18 (PrepPak 500 cartridge, Waters Corp., Milford, MA) using a gradient of 35–51% acetonitrile, 0.1% trifluoroactic acid over 25 min. Fractions containing protein were lyophilized and then dissolved in 25 mM Tris-HCl, pH 8.0, and further purified using ion exchange chromatography on a Perceptive Biosystems Biocad Sprint perfusion chromatography system using a Mono-Q anion exchange column (Amersham Pharmacia Biotech). The protein was eluted in a buffer containing 25 mM Tris-HCl, 1.0 mM EDTA/EGTA, pH 8.0, with a 0–0.5 M NaCl gradient at a flow rate of 5 ml/min. All buffers used in the purification of wild-type proteins contained 20 mM dithiotreitol to inhibit disulfide bond formation. Fractions containing protein were desalted and concentrated using a Millipore Ultrafree-4 centrifugal filter devices (5-KDa cut-off). Extinction coefficients were determined from 1 mg/ml samples of MRP8C42S and MRP14CBS by acid hydrolysis and amino acid analysis.

**Isoelectric Focusing—**Isoelectric focusing was performed on a Multiphor II isoelectric focusing system (Amersham Pharmacia Biotech) using Servalyt Precote polyacrylamide gels (SERVA, Heidelberg, Germany) and Pharmalyte 3–10. A 250 µl aliquot of a 20 mg/ml gel solution (Serva, Life Technologies, Inc.), \( \text{NH}_4\text{Cl} \) as the sole nitrogen source, and glucose as the carbon source. Ammpicillin was added to a final concentration of 100 µg/ml. 5 ml of an overnight culture grown in the same media. Cells were allowed to grow another 18–24 h before harvesting. Since the pET1120 plasmid contains no lac repressor gene, protein expression was not tightly controlled, and the system was leaky enough to produce large amounts of protein in rich media without the need for induction.

For \textsuperscript{15}N-labeled protein, cells were grown at 37 °C in M9 minimal media supplemented with ampicillin to a final concentration of 100 µg/ml. The solution was dialyzed using 18,000 × 10,000 MWCO dialysis membrane. The dialyzed solutions were added to 200 ml of a 0.5 M NaOH solution (5 mM acridine syringe filter (Gelman Sciences, Ann Arbor, MI) prior to further purification.

The MRP8s were isolated by preparative reverse-phase HPLC on a DeltaPak C18, 500 cartridge, Waters Corp., Millford, MA) using a gradient of 35–51% acetonitrile, 0.1% trifluoroactic acid over 25 min. Fractions containing protein were lyophilized and then dissolved in 25 mM Tris-HCl, pH 8.0, and further purified using ion exchange chromatography on a Perceptive Biosystems Biocad Sprint perfusion chromatography system using a Mono-Q anion exchange column (Amersham Pharmacia Biotech). The protein was eluted in a buffer containing 25 mM Tris-HCl, 1.0 mM EDTA/EGTA, pH 8.0, with a 0–0.5 M NaCl gradient at a flow rate of 5 ml/min. All buffers used in the purification of wild-type proteins contained 20 mM dithiotreitol to inhibit disulfide bond formation. Fractions containing protein were desalted and concentrated using a Millipore Ultrafree-4 centrifugal filter devices (5-KDa cut-off). Extinction coefficients were determined from 1 mg/ml samples of MRP8C42S and MRP14CBS by acid hydrolysis and amino acid analysis.

**Chemical Cross-linking—**The analysis of MRP8/MRP14 complex formation was carried out by using the bis(sulfoisocyanimidyl)suberate (Pierce) method described by Teigelkamp et al. (21). Purified recombinant MRP8 and MRP14 were diluted in phosphate-buffered saline at pH 8.5 to a final concentration of 50 µM. Cross-linking was initiated by the addition of freshly prepared bisulfosuccinimidylsuberate (20 µM stock solution) to a final concentration of 5 mM. Solutions were incubated at room temperature for 30 min before the addition of 1 mM Tris to quench the reaction. Cross-linked samples were incubated with β-mercaptoethanol prior to visualization on SDS-polyacrylamide gel electrophoresis.

**CD Spectroscopy—**Circular dichroism experiments were carried out on a Jasco J-810 spectropolarimeter with a 1-cm path length quartz cuvettes. Protein solutions (20 µM MRP8C42S, 20 µM MRP14CBS, and 10 µM MRP8C42S plus 10 µM MRP14CBS) were prepared in 1 mM Tris-HCl and 0.5 mM EDTA, pH 8.5. The solution was brought to 2.5 mM CaCl\textsubscript{2}, to obtain the spectra of the calcium-loaded state. Spectra were collected with an average time of 3 s for each point and a step size of 0.50 nm from 200 to 260 nm. All spectra were collected in triplicate and background-corrected against a buffer blank. A perl program (least-
ellip.pl) was written to determine the optimal least squares fit. Data were converted to mean residue ellipticity $\theta_m$ (degrees cm$^2$ dmol$^{-1}$) by using $\theta_m = \theta / (10,100)$, where $\theta$ is the measured ellipticity, $l$ is the cell path length in cm, $c$ is the molar concentration of protein in mol/liter, and $n$ is the number of residues/chain. The $\alpha$-helical content was estimated by measuring the mean residue ellipticity at 222 nm (44). Protein concentrations (20–25 $\mu$M) were determined by using the biochininic acid method (Pierce).

Fluorescence Spectroscopy—Measurements of the fluorescence excitation and emission spectra were conducted at 25 °C on a SLM-AMINCO Series 2 Spectrofluorometer equipped with a Hewlett Packard 5040 personal computer. Excitation spectra were measured from 250 to 320 nm with emission monitored at 335 nm. Scans were performed at 1 nm/s with the excitation and emission band pass set at 16 and 4 nm, respectively. Emission spectra were measured from 300 to 450 nm using an excitation wavelength of 290 nm. Scans were performed at 1 nm/s with the excitation and emission band pass set at 4 nm. Each 1.5-ml sample contained 5 $\mu$M total protein (MRP8C42S, MRP14C3S, or a 1:1 mixture) in 1 mM Tris-HCl, 0.5 mM EDTA at pH 8.5. For the calcium-loaded state, 1 mM CaCl$_2$ was added to a final concentration of 1.5 mM. All samples were preequilibrated overnight prior to the fluorescence measurements. Each experiment was collected in triplicate, and a perl program (lastgen.pl) was written to fit the data using a least squares algorithm.

NMR Spectroscopy—Wild type and mutant protein samples were dissolved and concentrated in a buffer containing 10 mM Tris-HCl, 100 mM KCl, and 5 mM EDTA/EGTA, at pH 8.5. For NMR samples of wild-type protein, deuterated dithiothreitol was also added to a final concentration of 5.0 mM. Pure $^2$H$_2$O was added to a final concentration of 10% for the spectrometer lock system. Spectra were recorded for the following samples: $^{15}$N-MRP8wt, $^{15}$N-MRP8C42S, $^{15}$N-MRP14C3S, $^{15}$N-MRP14wt, MRP8C42S + $^{15}$N-MRP14wt, MRP8C42S + $^{15}$N-MRP14C3S. The mixtures were made up in a 1:1.2 ratio of labeled to unlabeled protein (0.75–1.0 mM final concentration).

All NMR experiments were performed at 27 °C on a Bruker AMX500 operating at 499.67 MHz for $^1$H and 50.65 MHz for $^{15}$N. Phase-sensitive two-dimensional data were recorded using the method of States et al. (45). 1H chemical shifts were referenced to the H$_2$O peak at 4.75 ppm, and the $^{15}$N shifts were referenced indirectly using the $^1$H frequency of the H$_2$O resonance (46). The $^{15}$N-$^1$H HSQC spectra (47) were recorded with 16 transients/increment, a total of 128 complex points in $t_1$ and 512 points in $t_2$, and spectral widths of 1500.15 Hz in $w_1$ and 4000 Hz in $w_2$.

All two-dimensional data sets were processed on a Sun SPARCstation 1X workstation using FELIX (version 95.0; MSI, San Diego, CA). Homology Model of MRP8/MRP14—The model of apo-MRP8/MRP14 was generated using the Homology module of the InsightII package (MSI, San Diego, CA), based on the low resolution structure of the apo state of the related S100 protein calcyclin (29). Calcyclin is a symmetric homodimer, so the model was created with MRP8 aligned to one subunit and MRP14 aligned to the other. The automated sequence alignment algorithm in the software was used to define the conserved regions of MRP8 and MRP14. Residues 1–42 and 51–88 of MRP8 were assigned to residues 1–42 and 53–90 of calcyclin, respectively, and residues 4–45 and 57–96 of MRP14 were assigned to residues 1–42 and 53–90 of calcyclin, respectively. After construction of the conserved regions, the loop or "hinge" regions were generated for residues 43–50 of MRP8 and residues 46–56 of MRP14, and then the model was minimized using 3000 iterrations of steepest descents minimization in the Discover module of InsightII.

During minimization, the atomic coordinates for residues 1–42 and 51–88 of MRP8 and residues 4–45 and 57–96 of MRP14 were held fixed. The $\alpha$ angles within the hinge regions of MRP8 (residues 43–50) and MRP14 (residues 46–56) and the tail region of MRP14 (residues 97–114) were constrained to $-180^\circ$ throughout the minimization process. The model was considered sufficiently minimized when no van der Waals violations were detected.

**RESULTS**

**Cloning of MRP8 and MRP14**

In order to obtain sufficient quantities of protein for biophysical characterization and structure/function studies, the cDNA containing the entire coding sequence for human MRP8 and MRP14 was cloned into the bacterial expression vector PET1120. This expression vector was previously shown to efficiently overexpress the very closely related S100 protein calcyclin. The cDNAs were engineered with 5' NdeI restriction sites and 3' BamHI restriction sites for facile insertion into the expression vector downstream of the efficient T7 polymerase promoter. All gene sequences were confirmed by standard DNA sequencing methods.

MRP8 and MRP14 each contain a single cysteine residue. To avoid problems associated with the formation of unwanted disulfide bonds, the MRP8C42S and MRP14C3S mutant genes were also constructed and inserted into the PET1120 expression vector. DNA sequencing showed that the gene encoding MRP14C3S had a single base pair mutation (G to A) at position 318. However, both codons (AAG, AAA) code for the amino acid lysine, making this a silent mutation.

**Protein Expression**

The expression vectors containing the cDNA for MRP8 wt, MRP8C42S, MRP14wt, and MRP14C3S were used to transform the protease-deficient E. coli strain BL21(DE3). In rich media, BL21(DE3) cells transformed with the expression plasmids overexpressed proteins that migrated in SDS gels with apparent molecular masses of 8 kDa (MRP8wt, MRP8C42S) or 14 kDa (MRP14wt, MRP14C3S). Overexpression of MRP8 and MRP14 proved not to be detrimental to the bacteria, presumably because the proteins formed inclusion bodies (Fig. 1). Since these plasmids do not carry the lac repressor gene, high level expression of these proteins in rich (2× YT) media was attained without the need for induction. However, in M9 minimal media, protein expression was enhanced considerably after induction with isopropyl-thio-β-D-galactoside. In minimal media, cells were grown from mid- to late log phase and induced with isopropyl-thio-β-D-galactoside and then grown an additional 24 h before harvesting.

Approximately 100 mg of crude protein could be obtained from 1-liter cultures in 2× YT media, whereas expression levels dropped to nearly half in minimal media. Since tens of milligrams of purified, isotopically enriched protein is necessary for multidimensional NMR studies, the cost effectiveness of different carbon sources on expression levels in minimal media were explored. The choice of carbon sources (glycerol or glucose) made little difference to the overall expression levels in minimal media. Excess glucose was shown to increase the expression levels only slightly.

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2 This program is available on the World Wide Web at [http://chazin.scripps.edu/wisdom](http://chazin.scripps.edu/wisdom).

3 M. Lubienski, J. Glasser, and W. J. Chazin, unpublished results.
Purification

It was apparent early on that overexpression of MRP8 and MRP14 led to formation of inclusion bodies. In fact, no MRP8 (and small amounts of MRP14) was found in the cytosolic or Triton X-100 fractions after cell lysis (Fig. 1). This greatly facilitated the purification process. After treatment with Triton X-100, the inclusion bodies could be pelleted, and the recombinant protein could be isolated from the inclusion bodies by dissolving the pellet in 8 M urea. Spectroscopic analysis and dimerization assays (see below) indicated that dialysis of the urea solution resulted in properly folded protein. This is in agreement with experiments, which demonstrated that the protein complex was still recognized by a protein-specific antibody after unfolding in urea and refolding (24). Purification of recombinant MRP8 and MRP14 involved preparative scale reverse-phase HPLC followed by Mono-Q ion exchange chromatography. All four recombinant proteins eluted from C18 resin in nearly pure form with only minor amounts of contaminating proteins (data not shown). Ion exchange chromatography produced essentially pure MRP8 and MRP14, with each protein exhibiting a separate and distinct elution profile.

Characterization

Purified recombinant MRP8 and MRP14 were found to migrate in SDS-polyacrylamide gel electrophoresis with apparent molecular masses of 8 and 14 kDa, respectively (Fig. 1, lanes I and J). The molecular weights of MRP8wt, MRP8C42S, MRP14wt, and MRP14C3S were confirmed by either electrospray or MALDI mass spectrometry and were in close agreement with calculated molecular weights, determined using a JavaScript program. For MRP14wt and MRP14C3S, the calculated molecular masses were 130 atomic mass units higher than those determined by mass spectrometry. This discrepancy was attributed to the absence of the initiating methionine in both proteins, an apparent post-translational modification. Analytical isoelectric focusing was used to measure the isoelectric points of MRP8C42S (6.7) and MRP14C3S (5.5). Both of these values are in excellent agreement with the published values of 6.7 and 5.6, respectively, for the native MRPs (48).

Chemical Cross-linking—To determine the states of association of recombinant MRP8 and MRP14, chemical cross-linking experiments were employed using bis(sulfosuccinimidyl)suberate cross-linking agent. First, the possibility that the proteins form homodimers was explored. The Cys → Ser mutants were utilized for these experiments because this obviated the need for stringent methods to keep the proteins reduced. The results from the cross-linking experiments are clear in indicating that both MRP8 and MRP14 will form homodimers in homogenous solutions (Fig. 2). MRP14 is found to form only the monomer and the (MRP14)₂ complex. MRP8 is found to form monomer, homodimer, and a small amount of distinct, higher order aggregates (apparently trimer and tetramer).

The propensity for the formation of homodimers versus heterodimers was explored in a second set of experiments. These results showed a strong preference for the MRP8/MRP14 heterodimer in all solutions containing a mixture of both proteins (Fig. 2, lane A), which is similar to results obtained by Teigelkamp et al. (21). The formation of heterodimers was found to occur both in the absence or presence of Ca²⁺ (data not shown).

Circular Dichroism—The far UV CD spectrum of the wild-type and mutant MRP8 and MRP14 proteins indicates a high degree of α-helical content (Fig. 3), which is characteristic of the EF-hand family of proteins. The assignment of secondary structure and the examination of the calcium-dependent changes were made with the Cys → Ser mutants, because this obviated the need for reducing agents to keep unwanted disulfides from forming. The spectra for homogeneous solutions of MRP8 and MRP14 appear quite similar to the spectra obtained for 1:1 mixtures of the two proteins. Using the method of Chen et al. (44), an estimate of 44, 35, and 40% α-helix was obtained for MRP8C42S alone, MRP14C3S alone, and the mixture of
MRP8C42S plus MRP14C3S, respectively, in the presence of EDTA.

The spectra in Fig. 3 show that the addition of calcium ions to the MRP8/MRP14 heterodimer causes only very small changes in the degree of helicity. While a similar result is obtained for the homogenous solution of MRP14, there are surprisingly marked changes in the CD spectrum of MRP8 upon the addition of Ca$^{2+}$, with a reduction in signal intensity at 222 nm corresponding to an apparent 16% loss in helical content upon calcium addition. Although similar results have been reported for a few other S100 proteins (49, 50), we believe that in the case of MRP8, this observation is a consequence of calcium-induced aggregation of the protein, since higher concentrations of calcium ultimately lead to precipitation.

Fluorescence—The presence of a single tryptophan residue in both MRP8 and MRP14 provides a ready means to analyze these proteins by fluorescence spectroscopy. Trp$^{54}$ in MRP8 is located in the middle of helix III, and Trp$^{88}$ in MRP14 is located toward the COOH terminus of helix IV, both in their respective COOH-terminal EF hands. An excitation wavelength of 290 nm was selected to avoid excitation of the four tyrosine residues in MRP8 and the single tyrosine residue in MRP14. As for other biophysical experiments, the Cys → Ser mutants were utilized as opposed to the wild-type protein to minimize adverse affects due to the addition of reducing agents to the solution. The emission maxima of the spectra (Fig. 4) indicate that the tryptophan residues of both MRP8 and MRP14 are shielded from solvent, as the $\lambda_{\text{max}}$(emission) for free tryptophan is at 350 nm. It is notable that all tryptophan fluorescence spectra of MRP8 and MRP14 are asymmetric due to a small shoulder at $\sim$375 nm. The signal of free tryptophan is symmetric, and there is no readily apparent explanation for this shoulder.

In the spectra for the homodimer of MRP8 (Fig. 4A), there is a distinctive 20% drop in fluorescence emission intensity upon the addition of excess calcium ions. This is accompanied by a small shift in $\lambda_{\text{max}}$(emission) from 338 to 334 nm. In the MRP14 homodimer, a similar 17% reduction in fluorescence emission intensity is observed upon the addition of excess calcium ions but with only a slight shift in $\lambda_{\text{max}}$(emission) from 342 to 341 nm (Fig. 4B). These results indicate there are distinct Ca$^{2+}$-induced changes in the microenvironment of the respective tryptophan residues in both homodimers.

In the spectra for the 1:1 mixture of MRP8 and MRP14 (Fig. 4C), a significant but clearly smaller 7% drop in fluorescence emission intensity is observed upon the addition of excess calcium ions, accompanied by a small shift in $\lambda_{\text{max}}$(emission) from 338 to 337 nm. Thus, there appears to be some Ca$^{2+}$-induced change in the microenvironments of the two tryptophan probes.
However, since the fluorescence spectra of the MRP8/MRP14 heterodimer reflect the cumulative emission from the two different tryptophan probes, the results cannot be interpreted in a straightforward manner because it is not possible to attribute the relative contributions from each. Some insight can be obtained from the comparison of the spectrum of the heterodimer versus the two homodimers (Fig. 4C). The curves shown as dashed and thin solid lines were generated by adding the spectra for the MRP8/MRP8 and MRP14/MRP14 homodimers and then dividing by 2 to normalize the protein concentration. There is a very large difference in the calculated and observed fluorescence intensities, which strongly suggests that the microenvironments of one or both of the two probes in the heterodimer are different from their environments in the respective homodimers.

**NMR Spectroscopy**—The relative integrity of the MRP8 and MRP14 homodimers and the MRP8/MRP14 heterodimer were further examined by NMR spectroscopy. MRP8 has inherent solubility problems in the pH range of ~5.0–8.0, where most biomolecular NMR experiments are performed. This, in conjunction with the tendency of both MRPs to aggregate at the millimolar concentrations required for NMR, poses a significant challenge to NMR analysis. An extensive search of experimental conditions was required in order to obtain the spectra shown here.

The one-dimensional 1H NMR and two-dimensional 15N-1H HSQC experiments on the homogeneous solutions of both MRP8 and MRP14 in the absence of calcium exhibit poor sensitivity, broad resonance lines, and limited spectral dispersion. Such observations are typically associated with aggregation phenomena, limited structural stability, or conformational exchange. In contrast to the results obtained for the isolated proteins, the addition of MRP8 to the solution of MRP14 and of MRP14 to the solution MRP8 result in a radical improvement in resolution and signal dispersion in the spectra. Figs. 5 and 6 show the 15N-1H HSQC spectra of various mixtures of labeled and unlabeled MRP8 and MRP14. These spectra indicate that MRP8 and MRP14 preferentially form a stable, well packed heterodimeric complex.

The direct addition of calcium to the solutions of the homodimers and the heterodimer had different effects on each. MRP8 immediately forms an insoluble precipitate in direct proportion to the amount of Ca2+-induced conformational changes in the Ca2+-loaded MRP8/MRP14 heterodimer produced in this manner are clearly distinct from the spectrum of Ca2+-loaded MRP8/MRP14 homodimer, strongly suggesting that the Ca2+-loaded MRP8/MRP14 heterodimer is formed and that it has a unique structure (Fig. 7B).

**DISCUSSION**

The experiments presented here describe the high level expression and biophysical characterization of recombinant human MRP8 and MRP14, two members of the S100 calcium-binding protein family. Alignment of the calcium-binding loops of MRP8 and MRP14 with other members of the S100 family show that MRP14 contains the conserved sequence determinants necessary for calcium-binding in sites I and II that are found in other S100 proteins. MRP8 has a standard binding loop in site II but a significant Glu → Asp substitution at position 14 of the calcium-binding loop in the NH-terminal pseudo-EF hand (site I). Glutamic acid is highly conserved in this position in S100 proteins (Fig. 8) and the EF-hand calcium-binding protein family as a whole (51). This residue is known to play an important role in calcium binding by providing a bidentate ligand to the calcium ion and forming an integral part of a network of hydrogen bonds that serve to stabilize the binding loop (51). Substitution of this Glu residue is known to drastically reduce calcium affinity (52, 53) and alter the large Ca2+-induced conformational changes in the Ca2+ sensors calmodulin and troponin C (54, 55). In analogy to the S100 homologue p11, which also has an aspartate at this position, we anticipate that the NH-terminal site of MRP8 is not likely to bind Ca2+ with appreciable affinity.

One of the characteristic properties of S100 proteins is their tendency to dimerize. A calcium-dependent association of MRP8 and MRP14 has been reported (21, 48), suggesting the preferential formation of a 1:1 complex. In fact, the Kyte and Doolittle hydrophathy analysis (56) shown in Fig. 9 indicates that MRP8 and MRP14 have hydrophobic profiles similar to S100 proteins known to dimerize (57, 58). Both MRP8 and MRP14 exhibit the same hydrophobic NH2 and COOH termini and hydrophilic EF-hand regions (92) as calyculin and S100j. These results imply that both MRP8 and MRP14 should also form dimers.

The absence or presence and the biological significance of disulfide-linked covalent dimers of S100 proteins has been a topic of some debate (57, 58). It is not known whether MRP8 or MRP14 form biologically relevant, disulfide-linked dimers in solution. To examine this possibility, a homology model of the MRP8/MRP14 complex was assembled based on the three-
Dimensional solution structure of apo calcyclin determined in this laboratory (29). Inspection of this model suggests that the distance between Cys42 in MRP8 and Cys3 in MRP14 would make it highly unlikely that biologically relevant disulfides would form in the heterodimer (Fig. 10). This prompted the production of the two MRP mutants, MRP8C42S and MRP14C3S, which provide a means to avoid problems associated with unwanted disulfide bond formation, particularly during biophysical characterization at high protein concentrations. UV, CD, fluorescence, and especially NMR experiments showed that the mutant proteins are essentially identical to wild type.

The propensity of S100 proteins to dimerize and the pairing of MRP8 and MRP14 in vivo motivated the characterization of the extent to which these two proteins associate with each other. Chemical cross-linking experiments showed that homodimers (and for MRP8, specific higher order oligomers) are formed in solutions containing only MRP8 or MRP14. In mixtures of the two proteins, the heterodimer is greatly preferred (in a ratio of at least 10:1; Fig. 2). The uniqueness of the heterodimer is strongly supported by the observation of (i) very poor NMR spectral features for the homogenous solutions of either MRP8 or MRP14 but superior results for the 1:1 mixture (Figs. 5 and 6); (ii) no evidence for a second set of signals in any of the NMR spectra of the heterodimer, which suggests that the equilibrium concentration of homooligomers must be less than 1:50; (iii) NMR line widths and sensitivity in the two-dimensional 15N-1H HSQC spectra that are consistent with the formation of a dimeric (and not higher order) species. These results suggest a unique complementarity between MRP8 and MRP14, leading to a well folded stable heterodimer.

The comparison of the fluorescence emission spectra shown in Fig. 4 strongly suggests distinct differences between the structures of the homodimers and the heterodimer. The low fluorescence intensity of Trp88 in the MRP14/MRP14 homodimer (Fig. 4B) suggests that it is more solvent-exposed than is Trp54 in the MRP8/MRP8 homodimer (Fig. 4A). This suggests a rationale for why the co-addition of the normalized spectra of the homodimers does not correspond to the experimentally observed spectrum of the heterodimer. The sequence homologs of Trp88, Met82 in calcyclin, and Thr82 in S100β, are residues that are known to participate in the formation of a stable dimer interface (29, 59). If Trp88 is partially solvent-exposed in the homodimer but becomes buried in the well packed heterodimer, then the intensity of Trp88 fluorescence is expected to increase in the less polar environment of the heterodimer (47). The more highly sequestered location of Trp88 in the dimer interface is attributed to a greater degree of complementarity in the packing of the hydrophobic side chains in the heterodimer versus the homodimer. The significance of this hypothesis must now be explored by the combination of site-directed mutagenesis and more detailed structural analysis.

We have characterized the Ca2+ dependence of the structural properties of the MRP8, MRP14, and MRP8/MP14 dimers. The CD spectra show that calcium addition leads to
only slight changes in helicity for the MRP14 homodimer and the MRP8/MRP14 heterodimer. However, there is a distinct reduction in molar ellipticity in the spectrum of MRP8 upon calcium addition, similar to that seen for other homodimeric S100 proteins \((\text{e.g., S100A3 (50) and S100b (49)})\). However, in the case of MRP8, there are potential complications due to the limited solubility in the presence of calcium, such that the change in CD intensity could also arise from aggregation. In addition, changes in secondary structure upon calcium binding are not expected, since there is no change in the distribution of secondary structure seen upon Ca\(^{2+}\)-loading in the three-dimensional structures of calcyclin (60) and S100b (61, 62) or other EF-hand CaBPs (\text{e.g., Ref. 63}).

The fluorescence data show distinct changes in emission intensity upon calcium addition for all three dimers. This loss in intensity is consistent with that seen for calgranulin C and S100b (50, 64), suggesting that there are distinct calcium-induced structural changes for the MRP8/MRP8, MRP14/MRP14, and MRP8/MRP14 dimers. The presence of Ca\(^{2+}\)-induced changes is most clearly exemplified in the 15N-1H HSQC spectra of the Ca\(^{2+}\)-loaded MRP14/MRP14 homodimer and the MRP8/MRP14 heterodimer (Fig. 7, \(A\) and \(B\), respectively). Each spectrum is distinctly different from their respective apo counterparts (Fig. 6), which directly indicates that changes are brought on by Ca\(^{2+}\) binding. The ability to obtain spectra indicative of a stable well packed homodimer of MRP14, but not of MRP8, is an intriguing result in light of the hypothesis that MRP14 alone, but not MRP8 alone, has biologically relevant activity in specific inflammatory events.

In summary, the chemical cross-linking, CD, fluorescence, and NMR analysis of MRP8 and MRP14 show that a stable well packed heterodimeric complex is preferentially formed both in the absence and presence of calcium ions. These results indicate that the functionally relevant form of the MRP8 and MRP14 complex is a heterodimer. Furthermore, the corresponding studies of isolated MRP14 strongly suggest that its expression and functional roles independent of MRP8 are associated with a stable dimeric state. The determination of the three-dimensional solution structures of MRP8/MRP14 and MRP14/MRP14 is currently in progress in this laboratory.
These results should provide critical insights into the purported roles of the MRPs in calcium signaling pathways, as well as the interactions of these proteins with each other and with other proteins involved in the mechanisms underlying inflammatory disease.

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