Total Chemical Synthesis of Human Psoriasin by Native Chemical Ligation†

Xiangqun Li, Erik de Leeuw, and Wuyuan Lu*

Institute of Human Virology, University of Maryland Biotechnology Institute, 725 West Lombard Street, Baltimore, Maryland 21201

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ABSTRACT: Human psoriasin (S100A7), a member of the S100 family of calcium-binding proteins, is richly expressed in keratinocytes of patients suffering from psoriasis. To date, the exact physiological function of psoriasin abundant in many human cell types remains unclear. A recent report by Schröder and colleagues suggests that psoriasin, purified from human stratum corneum extracts, selectively kills *Escherichia coli* by sequestering Zn^{2+} ions essential for bacterial growth, indicative of an important role in innate immune defense against microbial infection. We chemically synthesized the N-terminally acetylated psoriasin of 100 amino acid residues using solid phase peptide synthesis in combination with native chemical ligation. More than 140 mg of highly pure and correctly folded synthetic psoriasin was obtained from a single synthesis on a 0.25 mmol scale. Analysis of synthetic psoriasin by size exclusion chromatography showed that the protein forms a homodimer in solution. Circular dichroism analysis indicated that the α-helicity of psoriasin increases by more than 20% in the presence of $CaCl_2$ or $ZnCl_2$, suggesting a metal ion binding induced conformational change. Circular dichroism based titration further established that the synthetic protein binds two Ca^{2+} and two Zn^{2+} ions per dimer, in agreement with the published structural findings. Importantly, the ability of the synthetic protein to kill *E. coli* and the inhibition of the killing by $ZnCl_2$ is comparable to that of psoriasin isolated from its natural source. The robust synthetic access to large quantities of human psoriasin should facilitate studies of its biological functions as well as its mode of action.

Human skin epithelium acts as a physical barrier separating the body from the environment. Although skin is constantly challenged by microbes, it is difficult to penetrate and shows a low rate of infection that can be attributed to the innate immune capability of skin keratinocytes to produce a battery of effector molecules warding off microbial invasion. Among these immune effector molecules produced by keratinocytes are inducibly expressed antimicrobial proteins with a broad spectrum of activity, such as human β-defensin 2 and 3 and the cathelicidin LL-37 (*1*−*3*).

In contrast to many species of bacteria, *Escherichia coli* is rapidly cleared from human skin (*4*). Recently, psoriasin was identified by Schröder and colleagues as the major *E. coli* killing molecule secreted by healthy skin keratinocytes (*5*). Psoriasin was first identified in psoriatic skin lesions showing an up-regulated expression (*6*). However, the expression of psoriasin is not limited to psoriatic skin as the protein is also expressed in carcinomatous tissues from the skin (*7*), bladder (*8*), stomach (*9*), and breast (*10*−*14*). While its biological function is presently not fully understood, psoriasin has been found to have chemotactic properties (*15*) and is thought to play an important role in innate immunity (*16*).

Psoriasin, a small protein of 100 amino acid residues, belongs to the S100 family of calcium-binding proteins (*17, 18*). Members of this protein family have been associated with a range of disorders including atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, Alzheimer's disease, and cancer (*19*). The S100 protein family comprises closely related, mainly α-helical acidic proteins, containing the conserved EF-hand motif, the most common calcium-binding motif found in proteins. Calcium binding induces a conformational change in most dimeric S100 proteins, which is thought to be important for structural stabilization and functional regulation of this family of proteins (*20*). Many S100 proteins also bind Zn^{2+} ions independently. Although not an essential structural element, Zn^{2+} enhances the binding affinity of S100 proteins for Ca^{2+} and/or for protein targets, thus playing a regulatory role in the action of these proteins (*21, 22*). In the case of psoriasin, Schröder and colleagues proposed that the protein kills *E. coli* by sequestering Zn^{2+} ions essential for bacterial growth (*5*), a mechanism that has yet to be fully scrutinized.

To further investigate the function of psoriasin and its mode of action, obtaining multimilligram amounts of the purified protein would be of great advantage. Isolation of the protein from its natural source requires a large amount of human material, which may not be readily available. Since naturally occurring psoriasin is N-terminally acetylated, in the absence of an efficient recombinant expression system capable of posttranslational modifications, we undertook the total chemical synthesis of psoriasin by means of native chemical ligation (*23, 24*), a robust and practical method for synthesizing small-to-medium sized proteins in large quantities. Here we describe the chemical synthesis, oxidative

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* To whom correspondence should be addressed. E-mail: luw@umbi.umd.edu. Tel: (410) 706-4980. Fax: (410)706-7583.

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folding, and purification of psoriasin, yielding 142 mg, on a 0.25 mmol scale of synthesis, of correctly folded and fully functional protein. Synthetic psoriasin is characterized by circular dichroism spectroscopy, mass spectrometry, and size exclusion chromatography. The effect of zinc on the structure and on the antimicrobial activity of synthetic psoriasin is also described.

MATERIALS AND METHODS

Materials. Boc-(L)-amino acids were purchased from Peptides International (Louisville, KY); Boc-Leu-OCH_2-PAM and Boc-Gln-OCH_2-PAM resin were obtained from Applied Biosystems (Foster City, CA); dichloromethane, *N*,*N*-dimethylformamide, and HPLC grade acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU)[1] was purchased from American Bioanalytical (Natrick, MA). Trifluoroacetic acid (TFA) was acquired from Halocarbon (River Edge, NJ) and hydrogen fluoride (HF) from Matheson Tri-gas (Montgomeryville, PA). *N*,*N*-Diisopropylethylamine (DIEA), thiophenol, and *p*-cresol were from Fluka (Switzerland), and ultrapure guanidinium hydrochloride was from ICN Biochemicals (Irvine, CA). *E. coli* ATCC 35218 was obtained from Microbiologics (St. Cloud, MN) and tryptic soy broth (TSB) from MP Biomedicals, Inc. (Solon, OH).

Solid-Phase Synthesis of Ps(1−*45)-*α*COSR and Ps(46*−*100).* The N-terminal peptide thioester Ps(1−45)-αCOSR (R = CH_2CO-Leu-OH) and the C-terminal fragment Ps(46−100) were synthesized, on appropriate resin (*25*), on an automated peptide synthesizer ABI 433A using an optimized in situ neutralization protocol developed by Kent and coworkers for Boc chemistry (*26*). After chain assembly, the N-terminal Boc group of Ps(1−45)-αCOSR-resin was removed with TFA and acetylated on resin with acetic anhydride. Side chain protecting groups were removed and peptides cleaved from the resin by treatment with anhydrous HF and *p*-cresol (9:1) at 0 °C for 1 h. Crude peptides were precipitated, washed with cold ether, and purified by preparative C18 reversed-phase (RP) HPLC and their molecular masses ascertained by electrospray ionization mass spectrometry (ESI-MS).

Native Chemical Ligation and Disulfide Bond Formation. Native chemical ligation between Ps(1−45)-αCOSR and Ps(46−100) was carried out in 0.1 M phosphate buffer, pH 7.4, containing 6 M guanidine hydrochloride and 2% thiophenol. The reaction proceeded to completion overnight, as monitored by analytical HPLC. The ligation product Ps(1−100) was purified by preparative RP-HPLC and its molecular mass verified by ESI-MS. Synthetic psoriasin was obtained through an overnight air oxidation of purified Ps(1−100) at 0.5 mg/mL in 0.1 M $NaHCO_3$, resulting in the formation of the only disulfide bridge in the protein.

Circular Dichroism (CD) Spectroscopy. Far-UV CD spectra were obtained on a Jasco J-810 spectropolarimeter at 25 °C using a 0.1-cm path length. The protein concentration was 20 μM in 20 mM HEPES buffer, pH 7.0, and the concentrations of $CaCl_2$ and $ZnCl_2$ each were 100 μM. Titration of 20 μM psoriasin at 222 nm by increasing amounts of Ca^{2+} or Zn^{2+} ranging from 0 to 100 μM was carried out in a 1-cm cuvette. Psoriasin was quantified by UV absorbance measurements at 280 nm using a molar extinction coefficient calculated according to the algorithm developed by Pace et al. (*27*). The α-helix content was estimated using the following equation described by Chen et al. (*28*):

$$\alpha\text{-helix } \% = ([\theta]_{222}/[\theta]_{max}) \times 100\%$$

where

$$[\theta]_{max} = -39500[1 - (2.57/n)]$$

and n is the number of residues.

Size Exclusion Chromatography. The association state of synthetic psoriasin was determined by measuring the relative molecular mass (M_r) by size exclusion chromatography on an Amersham Biosciences Superdex 75 column (10 × 300 mm) running 20 mM HEPES, 0.2 M NaCl, pH 7.0, at a flow rate of 0.5 mL/min. Five proteins, turkey ovamucoid third domain (OMTKY3) (5.6 kDa), ribonuclease A (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa) were chosen as standards. A calibration curve was obtained, i.e., $\log(M_r) = -0.093t$ (retention time) + 6.5351, from which the M_r value of synthetic psoriasin was derived. Reduced psoriasin dissolved in the HEPES buffer containing 1 mM DTT, and metal ion bound psoriasin in the presence of 100 μM Ca^{2+} and Zn^{2+}, were also subjected to SEC analysis under otherwise identical conditions.

Antibacterial Activity Assay. The antibacterial activity of synthetic psoriasin was tested using the colony counting assay developed by Steinberg and Lehrer (*29*). Typically, *E. coli* ATCC 35218 (1×10^5 CFU) was incubated, for 3 h at 37 °C, with synthetic, Zn^{2+}-free psoriasin in 10 mM phosphate buffer, pH 7.4, containing 1% TSB. Three 10-fold serial dilutions (×10, ×100, ×1000) of the incubation mixture were plated and incubated at 37 °C overnight. The antibacterial activity was determined by counting the number of CFUs on both sample and control plates. The influence of zinc on the antibacterial activity of psoriasin was analyzed using the same protocol except that psoriasin was pretreated with different concentrations of $ZnCl_2$ for 1 h at 0 °C.

RESULTS AND DISCUSSION

Total Chemical Synthesis via Native Chemical Ligation Produces Large Quantities of Synthetic Psoriasin in High Purity. N-Terminally acetylated psoriasin consists of 100 amino acid residues, among which a cysteinyl residue (Cys46) is conveniently located in the middle of the sequence, thus allowing for facile ligation of two separately synthesized peptide segments, Ps(1−45)-αCOSR and Ps(46−100) (Figure 1). Both peptides were synthesized on a 0.25-mmol scale using an optimized in situ neutralization protocol for Boc chemistry, yielding 340 mg (25%) and 445 mg (30%) of purified product, respectively. A representative native chemical ligation reaction on a 14 μmol scale nearly went to completion in 6 h, as monitored by analytical RP-HPLC

[1] Abbreviations: HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate,; TFA, trifluoroacetic acid; HF, hydrogen fluoride; DIEA, *N*,*N*-diisopropylethylamine; RP-HPLC, reversed-phase HPLC; ESI-MS, electrospray ionization mass spectrometry; CD, circular dichroism; SEC, size exclusion chromatography.

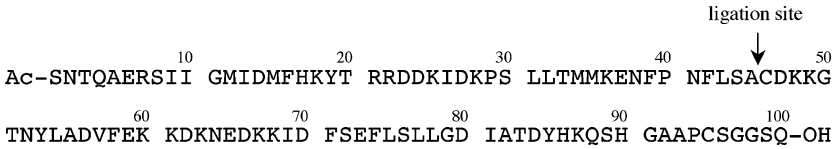

FIGURE 1: Amino acid sequence of N-terminally acetylated human psoriasin. The arrow denotes the site for native chemical ligation, i.e., Ala^{45}-Cys^{46}.

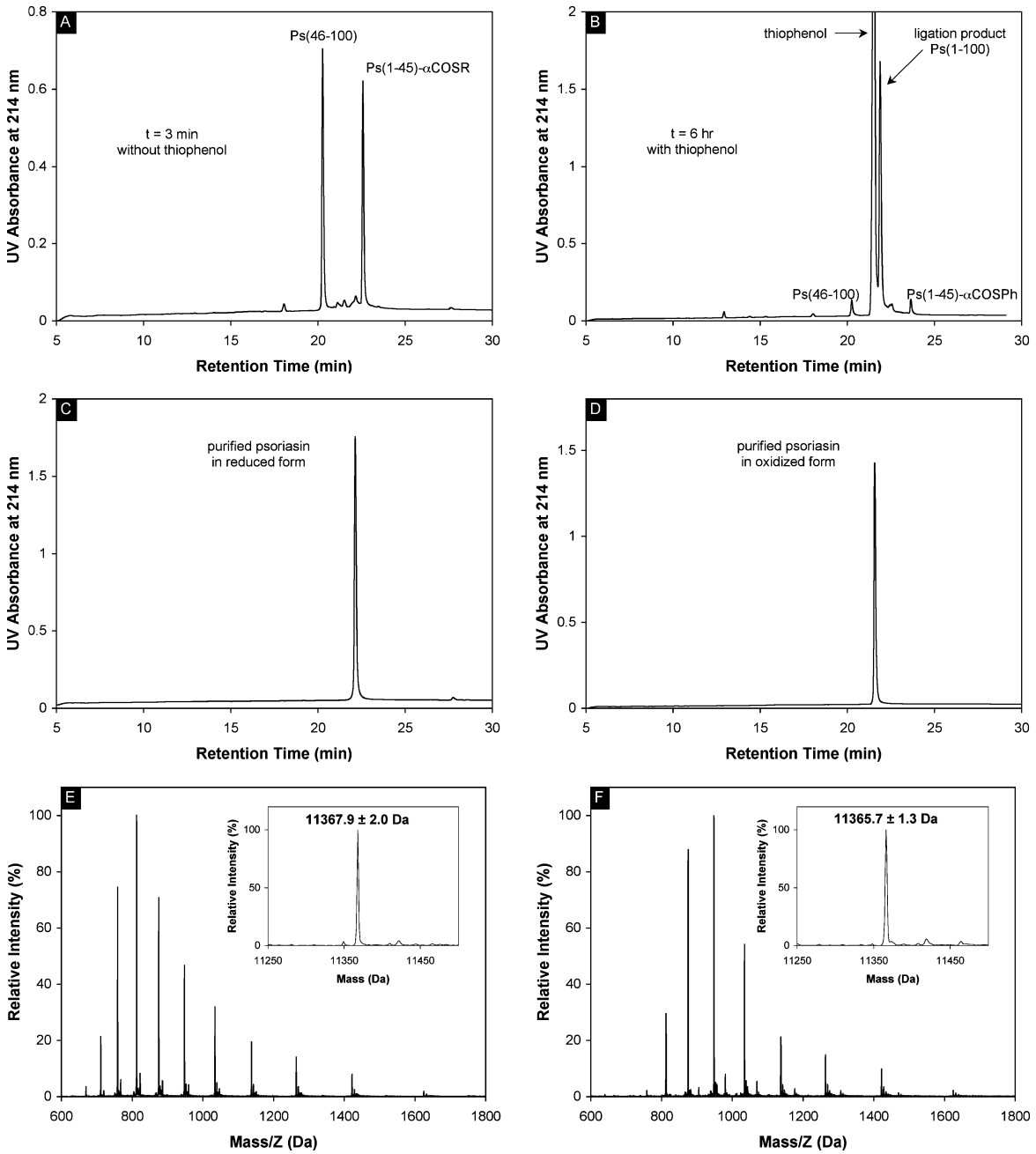

FIGURE 2: (A) Initial ligation of Ps(1−45)-αCOSR and Ps(46−100) prior to thiophenol addition. The reaction was monitored by analytical HPLC on a Waters Symmetry 300 C18 column (4.6 × 150 mm, 5 μm) running a 30-min gradient of 5 to 65% acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min. (B) Ligation reaction monitored after 6 h under identical chromatographic conditions. Ps(1−45)-αCOSPh is a transient intermediate derived from a nucleophilic substitution reaction of the reactant Ps(1−45)-αCOSR with the catalyst thiophenol. (C) Purified ligation product in reduced form on analytical C18 RP-HPLC. (D) Oxidized and folded psoriasin analyzed by analytical C18 RP-HPLC after purification. Note that the retention time of the oxidized psoriasin shifted slightly presumably as a result of the burial of hydrophobic residues. (E) Reduced psoriasin analyzed by ESI-MS. The molecular mass (11367.9 ± 2.0 Da) determined by ESI-MS agrees with the expected value of 11367.8 Da calculated based on the average isotopic compositions of the protein. (F) Oxidized and folded psoriasin analyzed by ESI-MS. The molecular mass (11365.7 ± 1.3 Da) is in agreement with the calculated value of 11365.8 Da, and the mass difference of 2.2 Da between the reduced and oxidized psoriasin results from the formation of the only disulfide in the protein. Note that the ESI charge envelope shifted as a result of disulfide formation, consistent with protein folding.

(Figure 2A,B). After HPLC purification, 74 mg of the full-length Ps(1−100) was obtained (Figure 2C), equivalent to a yield of 46%. To obtain the final product, Ps(1−100) was subjected to air oxidation and further HPLC purification

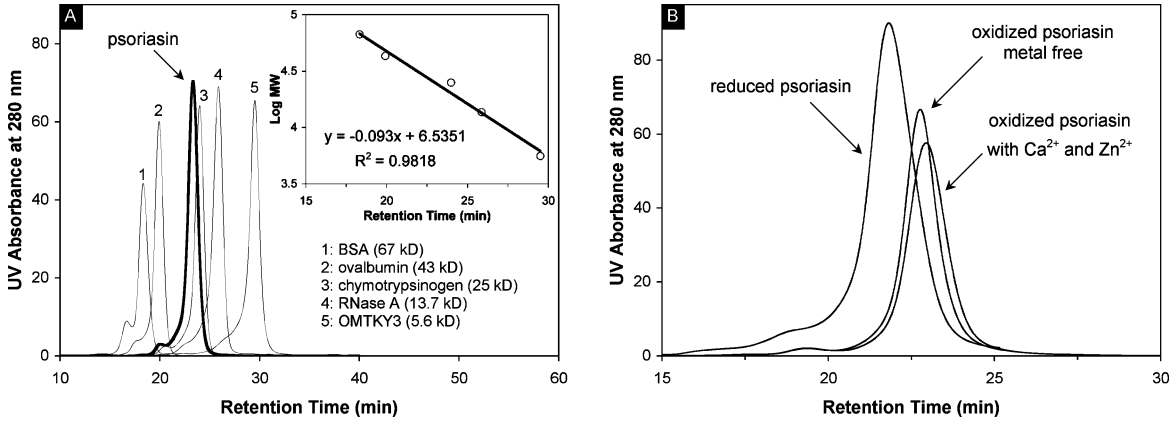

FIGURE 3: (A) A representative size exclusion chromatogram of synthetic psoriasin (thick line) and molecular mass standards (thin lines), monitored at 280 nm. A linear regression analysis of the correlation between logarithmic M_r and retention time is illustrated as the inset. Multiple injections were made, yielding fully overlapping chromatograms (data not shown). (B) Reduced psoriasin prepared in a DTT-containing sample buffer and metal loaded psoriasin in the presence of both Ca^{2+} and Zn^{2+} analyzed along with metal-free psoriasin by size exclusion chromatography.

(Figure 2D). A 0.25-mmol scale synthesis ultimately generated 142 mg of purified and correctly folded synthetic psoriasin, constituting an overall synthetic yield of 5%. Shown in Figure 2E,F is synthetic psoriasin, before and after oxidation, characterized by ESI-MS. The observed molecular masses (11367.9 ± 2.0 and 11365.7 ± 1.3 Da) were within experimental error of the expected values of 11367.8 and 11365.8 Da, respectively, calculated based on the average isotopic compositions of the protein. The characteristic shift in observed molecular mass, HPLC retention time, and ESI charge envelope between reduced and oxidized psoriasin was consistent with the formation of one disulfide bridge in and the folding of the molecule.

Native chemical ligation, a technique pioneered by Kent and colleagues, has allowed us to establish an efficient and robust synthetic access to large quantities of highly pure and correctly folded human psoriasin. This should significantly facilitate studies of structure and function relationships and of mechanisms of action for this protein. It should be noted that total chemical synthesis of another member of the S100 protein family, S100A1, was previously reported by Miranda et al. (*30*). The 91-residue protein contains no Cys and was manually synthesized stepwise using the same coupling chemistry originally developed by Kent and colleagues (*26*). The synthetic S100A12 dimerized in solution, was shown to bind Ca^{2+} and Zn^{2+}, and chemoattracted neutrophils and macrophages (*30*).

Synthetic Psoriasin Dimerizes in Solution. Under physiological conditions, most S100 proteins including psoriasin form dimers, with the exception of Calbindin D9k, which is shorter than average (78 residues versus 90−100) (*31*−*33*). Dimerization of various S100 proteins has been supported by structural analysis using X-ray crystallography and NMR spectroscopy. To examine the association state of synthetic psoriasin, the protein was analyzed by size exclusion chromatography. Shown in Figure 3A is synthetic psoriasin along with five molecular mass standards: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), ribonuclease A (13.7 kDa), and turkey ovomucoid third domain (5.6 kDa). Psoriasin eluted from the column, clustering with the chymotrypsinogen peak. A calibration curve was obtained (Figure 3A inset), from which an apparent molecular mass of 22.9 kDa was derived for

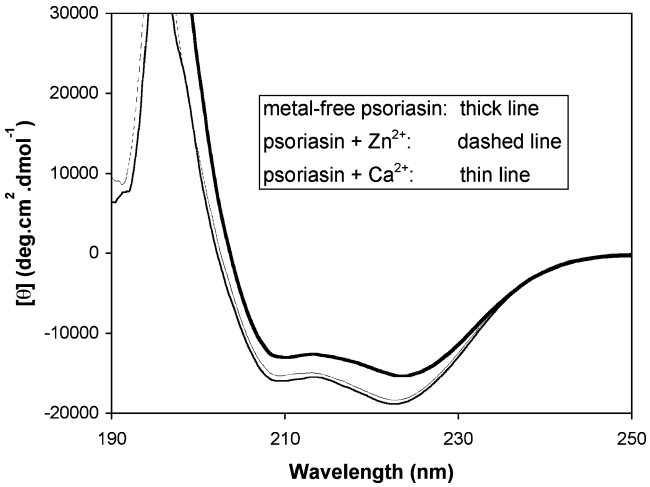

FIGURE 4: Circular dichroism spectra of 20 μM synthetic psoriasin in the absence of metal ions (thick line) and in the presence of 100 μM Zn^{2+} (thin line) or 100 μM Ca^{2+} (dashed line). The measurements were performed in 20 mM HEPES buffer, pH 7.0, at 25 °C. Note that the double minima at 208 and 222 nm are characteristic of an α-helical conformation, and the α-helicity increased with the addition of metal ions.

psoriasin, in agreement with the predicted value of a homodimer of 22.7 kDa.

S100 proteins dimerize in aqueous solution independent of metal binding. Wilder et al. demonstrated by NMR spectroscopy that the coordination of Zn^{2+}, which involves two residues from each subunit of rat S100B, brings the subunits closer to give a more compact dimer interface in Zn^{2+}−Ca^{2+}−S100B when compared to Ca^{2+}-bound S100B (*21*). Similar contraction around the Zn^{2+} binding sites was also observed for psoriasin (*34*). This Zn^{2+}-induced structural change likely explains our finding that in the presence of both Ca^{2+} and Zn^{2+} the size exclusion volume of synthetic psoriasin is slightly larger than that of a metal-free protein (Figure 3B). This is understandable because the metal-bound psoriasin is structurally more compact than its metal-free form (*34*, *35*), and consequently, its retention on size exclusion chromatography is longer due to a decreased solvent-accessible radius. Reduced synthetic psoriasin also dimerized in solution (Figure 3B), suggesting that disulfide formation has little impact on protein dimerization. This

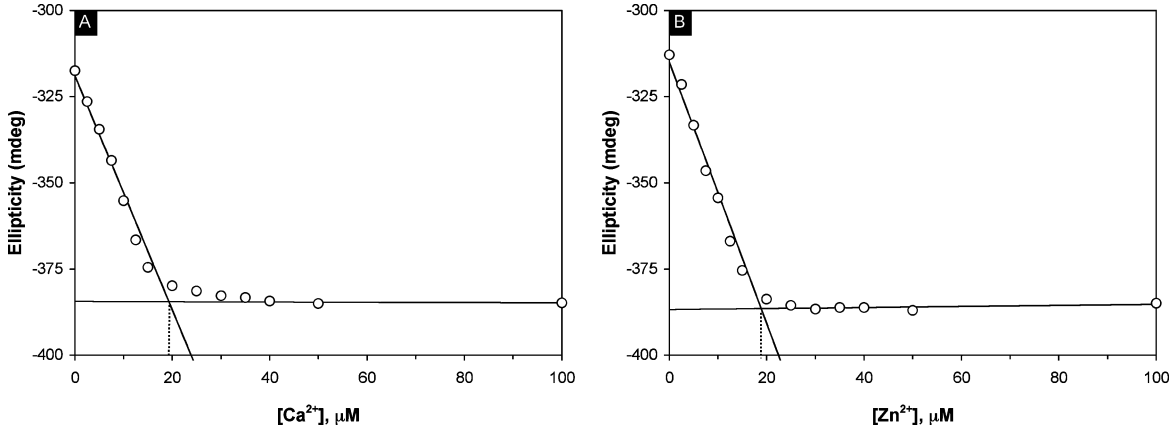

FIGURE 5: Titration of 20 μM psoriasin in 20 mM HEPES, pH 7.0, by Ca^{2+} (A) and by Zn^{2+} (B) monitored at 222 nm. The crossing point between two lines corresponds to a saturated binding of Ca^{2+} or Zn^{2+} by psoriasin, which is approximately at 20 μM.

finding is consistent with the fact that most S100 proteins contain no disulfide bonds. Not surprisingly, reduced synthetic psoriasin eluted with a noticeably smaller exclusion volume, indicating that the reduced protein is structurally significantly less compact than its oxidized counterpart.

Binding of Ca^{2+} or Zn^{2+} to Psoriasin Is Stoichiometric and Induces a Conformational Change. Structural studies indicate that binding of Ca^{2+} and/or Zn^{2+} causes local as well as global conformational rearrangements in S100 proteins. Since S100 proteins adopt similar structures rich in α-helices, metal binding induced conformational change can be readily detected by circular dichroism spectroscopy. To examine the effect of Ca^{2+} or Zn^{2+} (100 μM) on the solution structure of psoriasin (20 μM), CD spectra were acquired in the absence or presence of either divalent cation. Synthetic psoriasin in the metal-free state showed a predominantly helical structure in aqueous solution at neutral pH (Figure 4), exhibiting double minima at 208 and 222 nm characteristic of α-helix. The α-helical content of psoriasin was 39% as calculated by the $[\theta]_{222}$ (see Materials and Methods). Although the observed α-helical content (approximately 60%) is high in the crystal structure of psoriasin (*34*, *35*), a low percentage of α-helix calculated based on CD data is quite common for S100 proteins due to a dynamic nature of these molecules in aqueous solution (*30*, *36*, *37*). Upon addition of Zn^{2+} or Ca^{2+}, the helicity increased to 47% and 49%, respectively, demonstrating that the presence of Zn^{2+} or Ca^{2+} induced a conformation change in psoriasin, consistent with the published structural studies (*34*, *35*). Interestingly, the presence of both cations did not contribute additively to further increase in the α-helical content (data not shown).

Most S100 proteins contain two independent EF-hand calcium-binding motifs in each subunit, a canonical EF-hand in the C-terminal region and an S100-specific pseudo EF-hand at the N-terminus. However, for psoriasin a three-residue deletion in the N-terminal EF-hand loop region debilitates the protein to bind only one Ca^{2+} ion per monomer or two Ca^{2+} ions per dimer in the C-terminal EF-hand motif. By contrast, there exist two symmetrical Zn^{2+} binding sites in a dimeric psoriasin, each constituted by His86 and His90 from one monomer and His17 and Asp24 from the other (*34*).

The metal-induced increase in the α-helicity or decrease in the ellipticity of synthetic psoriasin is concentration

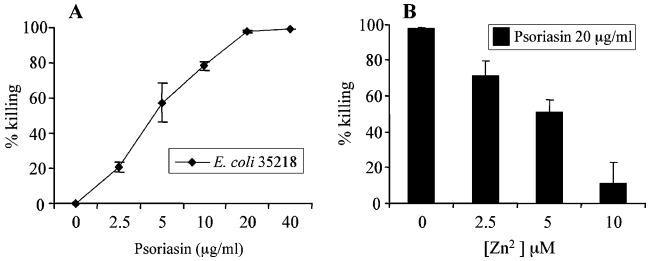

FIGURE 6: Antimicrobial activity of synthetic psoriasin against *E. coli*. (A) Dose-dependent antimicrobial activity of psoriasin against *E. coli* ATCC 35218 in the colony counting assay. Percentage of killing is expressed relative to bacteria incubated with buffer alone for 3 h at 37 °C. (B) Bactericidal activity of psoriasin is inhibited by Zn^{2+}. Psoriasin (20 μg/mL) was preincubated on ice for 1 h with the indicated concentrations of $ZnCl_2$. Antimicrobial activity was analyzed by the colony counting assay. Percentage of killing is expressed relative to bacteria incubated with buffer alone. Experiments were carried out three times, each in triplicate. Data presented are the mean ± SD of one representative experiment.

dependent on Ca^{2+} or Zn^{2+}. This afforded us a convenient way to determine the stoichiometry of the interaction between synthetic psoriasin and the metal ions by titrating the protein using an increasing amount of Ca^{2+} or Zn^{2+}. Shown in Figure 5 are the titration curves obtained at 222 nm with 20 μM psoriasin and a varying concentration of Ca^{2+} or Zn^{2+} ranging from 0 to 100 μM. As expected, the ellipticity linearly decreased in the low concentration range (0−20 μM), and, upon reaching the saturation point (~20 μM for both Ca^{2+} and Zn^{2+}), remained unchanged as the metal ion concentration further increased (20−100 μM). Clearly, synthetic psoriasin binds to Ca^{2+} or Zn^{2+} at a 1:1 molar ratio or two of each metal ion per dimer, consistent with the results obtained from structural studies of this protein (*34*).

Synthetic Psoriasin Kills E. coli, and Its Bactericidal Activity Is Inhibited by Zn^{2+}. In a recent study by Schröder and colleagues, psoriasin was identified as the main *E. coli* killing peptide secreted by keratinocytes (*5*). Although microbicidal activity was reported against other microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermis*, psoriasin showed most potent killing of several *E. coli* strains including ATCC strain 35218 (90% lethal dose, LD90 = 0.5 μM). In addition, the same study reported that Zn^{2+} inhibits the microbicidal activity of psoriasin. Therefore, the antimicrobial activity of synthetic psoriasin against *E. coli* ATCC 35218 was tested in the absence or presence of $ZnCl_2$ using standard colony-

counting assay (*29*). In the absence of Zn^{2+}, a dose-dependent antimicrobial activity was observed, with a LD90 of 15 μg/mL, or 1.3 μM (Figure 6A), comparable to the reported value of 0.5 μM for the same strain. Notably, the LD50 value (0.3 to 0.4 μM) determined in this work is identical to that reported by Gläser et al. (*5*). When psoriasin (20 μg/mL) was preincubated with different concentrations of $ZnCl_2$ before the antimicrobial assay, a dose-dependent inhibition of killing was observed (Figure 6B). We found that 10 μM Zn^{2+} abolished 90% of the bactericidal activity of psoriasin, in quantitative agreement with the results described previously (*5*).

Zinc sequestration by psoriasin has been proposed as one possible mechanism for microbial killing (*5*). Zn^{2+} ions inhibited the bactericidal activity of psoriasin in a dose-dependent manner. In addition, a low molecular weight Zn^{2+} chelator also effectively killed *E. coli* and the bactericidal activity was additively enhanced when used in combination with psoriasin. Furthermore, the antimicrobial activity of other members of the S100 protein family, such as the S100A8/A9 heterodimer (calprotectin) (*38*, *39*), is modulated by zinc. In the case of calprotectin, however, Murthy et al. demonstrated that the calprotectin-mediated candidastasis did not result from a direct zinc sequestration (*40*). In addition, psoriasin is among the weakest zinc-binding proteins in the S100 family (*21*).

In general, S100 proteins are considered primarily as zinc-regulated proteins (*41*−*43*). The crystal structure of Ca^{2+}-bound psoriasin has been resolved both in the Zn^{2+}-loaded and in the Zn^{2+}-free state (*34*). The binding of zinc not only causes a local structural change but also profoundly impacts the overall protein conformation (*34*). The location of the zinc-binding site in psoriasin is in close proximity to the putative substrate-binding cleft for S100 proteins, suggesting that zinc binding may regulate the interaction of psoriasin with receptor molecules. Thus, it may be conceivable that psoriasin acts against *E. coli* by interacting directly with an as yet unidentified component. This interaction may depend on a change in its conformation or affinity controlled by its zinc load. However, whether or not psoriasin exhibits, in addition to the suggested zinc sequestration mechanism, an alternative microbial killing mechanism remains to be investigated. To better understand the mode of action of psoriasin in microbial killing, mutational studies are obviously needed. In this regard, our facile synthetic access to human psoriasin should provide a useful tool for the elucidation of the molecular basis of how this protein works.

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