Maspin is a tumor suppressor protein expressed by normal human mammary epithelium but not by many breast tumor cell lines. Recombinant human maspin (rMaspin) inhibits tumor cell motility, invasion, and metastasis and thus has potential value as an anti-cancer therapeutic. Maspin is a member of the serpin family and, although the molecular mechanism by which maspin acts is unknown, recent work suggests that tissue plasminogen activator is a potential target. A puzzling observation in previous cell culture studies was loss of rMaspin activity at higher protein concentrations. One hypothesis to explain these results is self-association of rMaspin at the higher concentrations, which would be consistent with the tendency of serpins to form noncovalent polymers. This hypothesis is addressed by examining the relationship between rMaspin stability and self-association. Urea denaturation of rMaspin at pH 7 and 25 °C and at protein concentrations ranging from 0.01 to 0.2 mg/ml has been monitored by circular dichroism and intrinsic tryptophan fluorescence. Denaturation profiles show a protein concentration dependence and indicate the presence of at least one unfolding intermediate. The results suggest that destabilization of native monomeric rMaspin leads to partial unfolding and formation of an intermediate which can self-associate.

Maspin is a tumor-suppressing protein (M, 42,138) that was originally identified in normal human breast epithelial and myoepithelial cells (1). Maspin has subsequently been localized to epithelial and myoepithelial cells in a variety of tissues (2). Maspin expression is decreased or lost in most breast carcinoma cell lines and tissue (1) and its expression is down-regulated in metastatic prostate carcinoma cells as well (3, 4). Overexpression of maspin in breast carcinoma cell lines leads to decreased cell motility, invasiveness, and metastasis (1). These results suggest that maspin plays a key role in inhibiting the progression of breast cancer and, possibly, prostate cancer.

One very interesting observation with regard to both the mechanism and possible therapeutic potential of maspin is that addition of exogenous recombinant maspin (rMaspin) to cultured breast carcinoma cells lines leads to decreased cell motility and invasiveness (5–7). These results are in line with recent studies of conditioned medium from myoepithelial cell cultures: this medium inhibits motility and invasiveness of breast cancer cells and these activities are lost after removal of maspin from the medium (8, 9). Naturally occurring maspin and rMaspin thus must act at an extracellular site or be transported across the plasma membrane to an intracellular site of action. One possible target for maspin is tissue-type plasminogen activator (tPA) (10), although there is some disagreement on this point (11). Nevertheless, the fact that exogenous rMaspin is active suggests that rMaspin holds promise as a potential anti-cancer therapeutic.

The inhibitory effect of rMaspin on cancer cell motility and invasiveness shows a curious dose-response relationship: activity increases with increasing rMaspin concentration up to about 0.2 μM protein and then falls with further increases in protein concentration (5). One possible explanation for this behavior may be found in an analogous effect of rMaspin on tPA in vitro, where concentrations of rMaspin below 0.5 μM inhibit fibrinogen-activated tPA while higher concentrations stimulate tPA activity (10). However, a role for tPA in breast cancer cell motility and invasion has not yet been established. Another hypothesis to explain the curious concentration dependence of rMaspin activity in cell culture is self-association of rMaspin (5). This hypothesis is supported by the fact that maspin belongs to the serpin family and members of this family show a tendency to self-associate (e.g. Refs. 12–15).

Self-association of serpins generally involves a structural feature known as the reactive-site loop (RSL) and one of the three β-sheets, A through C, found in all serpins (16). The RSL of one molecule can insert into either the A or C sheet of another molecule (12). A related intramolecular association occurs between the RSL and the A-sheet of many serpins as part of an extraordinary conformational change from a metastable biologically active state (also known as the stressed or S state) to a more stable latent state (also known as the relaxed or R state).

rMaspin does not undergo the S to R transition, but results of electrophoretic studies suggest that rMaspin self-associates when subjected to mild perturbations in solution conditions (11). The goal of the present study is to develop a better understanding of the relationship between the structure and anticancer activity of rMaspin by focusing more closely on the relationship between stability and self-association of rMaspin. Urea denaturation of rMaspin at a variety of protein concentrations has been monitored by circular dichroism (CD) and fluorescence spectroscopy. The solution structure of rMaspin in the absence of urea has been investigated by analytical ultracentrifugation at 25 and 40 °C. Results of the present study demonstrate that native rMaspin at pH 7.0 is monomeric but that urea denaturation of rMaspin is a multistate reaction in which an intermediate state undergoes self-association.
**Unfolding and Self-association of Maspin**

### EXPERIMENTAL PROCEDURES

**Materials—**rMaspin was purified from *Saccharomyces cerevisiae* as described previously (11). rMaspin is >95% pure as judged by SDS-polyacrylamide gel electrophoresis. Biological activity of the same batch of rMaspin has been demonstrated in cell culture studies (7). Crystallized and ultrapure urea was purchased from Roche Molecular Biochemicals (Indianapolis, IN). All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ) or EM Science (Gibbstown, NJ) and were reagent grade or better. Water was deionized and glass-distilled. All buffer solutions were passed through 0.45-μm filters and degassed.

**Analytical Ultracentrifugation—**Sedimentation equilibrium experiments were conducted on a Beckman XL-I analytical ultracentrifuge equipped with an An-60 Ti rotor. Six-channel equilibrium centerpieces were used for protein concentrations of 9.5 μM (0.4 mg/ml) and 36 μM (1.5 mg/ml) were examined at pH 5.0, 6.0, and 7.0. The pH 6.0 and 7.0 solutions contained 50 mM sodium phosphate and 0.1 mM NaCl while the pH 5.0 solutions contained 50 mM sodium acetate and 0.1 mM NaCl.

Absorbance was monitored at 280 nm in all samples. Samples at 25 °C were centrifuged at 10,000, 12,000, and 17,500 rpm and those at 40 °C were centrifuged at 10,000 and 17,500 rpm. These samples were then subjected to 40,000 rpm to deplete the meniscus of protein, which facilitates measurement of baseline absorbance values. Data were analyzed using the ORIGIN™ software (version 3.78, Microcal Software, Northampton, MA) supplied with the ultracentrifuge and running on an IBM PC. The partial specific volume, \( \bar{v} \), of rMaspin was calculated to be 0.716 ml/g on the basis of amino acid composition (17). The calculated solution density, \( \rho \), for the pH 5.0, 6.0, and 7.0 samples was 1.004, 1.007, and 1.008 g/ml, respectively (18). The apparent molecular weight of rMaspin, \( M_r \), was determined by fitting absorbance, \( A \), as a function of radial position, \( r \), to the following equation for an ideal solution containing a single species (19),

\[
A(r) = A_0 \exp\left[M(1 - \bar{v}r^2)/2(\bar{v}^2 r^2 - \bar{v}^2/2RT)\right] + A_0 \tag{1}
\]

where \( r_0 \) is the radial position of the meniscus, \( \omega \) is the angular velocity of the rotor, \( R \) is the gas constant, \( T \) is the absolute temperature, and \( A_0 \) is the baseline absorbance in the absence of protein.

**Circular dichroism spectroptosimetry—**CD data were acquired on an AVIV 62DS spectropolarimeter equipped with a thermoelectric temperature controller. Protein solutions were passed through 0.45-μm filters and degassed prior to data acquisition. Far-UV CD spectra were obtained at 25 °C with samples containing 5 mM sodium phosphate and 4.8 μM (0.20 mg/ml) rMaspin. A 0.1-cm path length cuvette was used, spectra were collected at 0.5-nm intervals with an averaging time of 2 s and two scans were averaged.

**Fluorescence Spectroscopy—**Fluorescence data were obtained on a Fluorolog-3 spectrofluorometer (Jobin Yvon-Spex Instruments, Edison, NJ) equipped with a circulating water bath for temperature control. Intrinsic tryptophan fluorescence was excited at 295 nm. Excitation and emission band widths were 2.5 nm, data were collected at 1-nm intervals, and two scans were averaged for each spectrum.

**Chemical Denaturation—**Urea denaturation experiments were done at pH 7.0 with solutions containing 100 mM sodium phosphate, 1 mM dithiothreitol, 1 mM Na2-EDTA and the indicated concentration of urea. Buffer solutions containing approximately 9 M urea were prepared just before use. The concentration of rMaspin was determined using a calculated extinction coefficient for native rMaspin of 18,450 M⁻¹ cm⁻¹ at 280 nm (21).

The same protein solutions were used for CD and fluorescence measurements of urea denaturation. For CD measurements at 222 nm, a 1-cm path length cuvette was used for solutions containing 0.24 and 1.2 μM rMaspin and a 0.2-cm path length cuvette was used for 4.8 μM solutions of rMaspin. The averaging time was 2 s and the reported signal is the mean for data collected over a 2-min period. Fluorescence spectra were acquired as described above. The intensity weighted average emission wavelength (22) was calculated from emission spectra collected from 305 to 500 nm.

**RESULTS**

**Analytical Ultracentrifugation—**One hypothesis to explain the loss of rMaspin activity at concentrations greater than 0.2 μM is formation of biologically inactive multimers. This hypothesis was tested using equilibrium analytical ultracentrifugation. The results suggest that, in the absence of other contributing factors, the hypothesis may be incorrect: at pH 7.0, native rMaspin is monomeric at concentrations approaching 20 μM at both 25 and 40 °C (Fig. 1). Solutions containing higher concentrations of rMaspin show clear evidence for non-ideality (data not shown), leading to apparent molecular weights that are less than expected on the basis of sequence data.

In cell culture studies, the pH of the medium declines over time as a consequence of cellular metabolism. To investigate the possibility that such a decrease in pH leads to self-association of rMaspin, the protein was subjected to analytical ultracentrifugation at more acidic pH. The results at pH 6 are similar to those seen at pH 7; native rMaspin is monomeric (Fig. 1). At pH 5 and 25 °C, rMaspin forms a visible precipitate at all concentrations and the solutions are not suitable for quantitative analysis of sedimentation behavior. Acidic pH thus leads to self-association of rMaspin. However, the pH of cell culture medium is not likely to fall below pH 6, so we conclude that acidification of cell culture medium is not sufficient to drive self-association of rMaspin.

**Chemical Denaturation—**The centrifugation experiments were conducted in simple phosphate or acetate buffer solutions containing 100 mM NaCl, while cell culture medium contains a host of other components that may contribute to self-association.
Results of previous studies demonstrated that rMaspin is indeed capable of undergoing self-association at moderately acidic or basic pH, or at pH 7.4 and temperatures greater than 40 °C (11). However, these studies explored a relatively small set of solution conditions, so chemical denaturation has been used in the present study to obtain a more complete and precise understanding of rMaspin stability and self-association.

Urea denaturation of rMaspin at concentrations ranging from 0.24 to 4.8 mM has been monitored by both far-UV CD at 222 nm (Fig. 2A) and intrinsic tryptophan fluorescence (Fig. 2B) at pH 7 and 25 °C. The urea denaturation profiles monitored by CD and fluorescence are in qualitative agreement on two major points: the denaturation profiles are sensitive to protein concentration and at least two distinct transitions are observed at rMaspin concentrations >0.24 mM. The chemical denaturation of rMaspin must thus involve at least three states: native (N), an intermediate (I), and the denatured or unfolded (U) state.

The ultracentrifugation experiments show that N is monomeric in these solution conditions, so chemical denaturation has been used in the present study to obtain a more complete and precise understanding of rMaspin stability and self-association.

Unfolding and Self-association of Maspin

The simplest model that is consistent with the data is as follows,

\[ nN \rightleftharpoons [nI + I] \rightleftharpoons U \]  

(Eq. 2)

where \( n \) is the stoichiometry of the association reaction, which is not yet known. The square brackets indicate an equilibrium between monomeric and multimeric I that could be either on or off the direct pathway for unfolding. In either case, this model predicts that the transition from N to I should occur at lower urea concentrations with increasing protein concentration. This is not evident from the CD data in Fig. 2A, but changes in the transition midpoints may be masked by the complexity of the denaturation profiles.

Ideally, least squares analysis of the denaturation profiles would be used to obtain estimates for the thermodynamics of rMaspin denaturation and self-association. In the case of rMaspin, this type of analysis is confounded at present by a lack of knowledge concerning the stoichiometry of self-association and by incomplete reversibility of denaturation: dilution of rMaspin out of 8 M urea only leads to partial recovery of the spectroscopic signals (Fig. 3). The extent of recovery depends on protein concentration, so the irreversible reaction involves self-association of rMaspin.

The stability of rMaspin has been measured at 37 °C to investigate the possibility that partial unfolding and self-association are more likely at the temperature of cell culture studies. Urea denaturation of 1.2 mM rMaspin at pH 7.0 and 25 °C (Fig. 4) shows at least two interesting differences relative to data obtained at 25 °C. First, the midpoint of the initial transition occurs at a lower urea concentration, about 1.6 M at 37 °C versus about
1.9 M at 25 °C, suggesting that native rMaspin is less stable at the higher temperature. Second, the midpoint of the second transition occurs at a higher urea concentration, about 3.5 M versus approximately 3 M at 25 °C. The partially unfolded and multimeric I state of rMaspin is thus stabilized at the higher temperature. Overall, rMaspin is more likely to undergo partial unfolding and self-association at 37 °C than at 25 °C.

CD Spectra—The conformational changes associated with the denaturation and self-association of rMaspin have been investigated by CD and fluorescence. Urea denaturation monitored by far-UV CD suggests that the transition from N to I involves a significant loss of secondary structure (Fig. 2). Comparison of far-UV CD spectra in the presence and absence of urea further substantiates this conclusion (Fig. 5A).

In the absence of urea, the CD spectrum is dominated by a minimum at about 222 nm and a maximum at 196 nm. A molecular model for native rMaspin has been generated on the basis of its homology to ovalbumin (23). The predicted secondary structure content is approximately 30% helix, 30% β-strand, 10% β-turn, with the balance in irregular structure. These values have been used as input into Equation 11 of Yang and co-workers (24) to predict mean residue ellipticities for native rMaspin. Predicted values of \( \pm 12,000 \) and \( +20,000 \) deg cm\(^2\) dmol\(^{-1}\) at 222 and 196 nm, respectively, are similar to the observed values of \( -12,500 \) deg cm\(^2\) dmol\(^{-1}\) and \( +16,000 \) deg cm\(^2\) dmol\(^{-1}\). CD data are thus consistent with the homology model for rMaspin.

rMaspin in 8 M urea has no detectable secondary structure as measured by CD (Fig. 5A). The CD spectrum under conditions where the intermediate, I, is well populated suggests a loss of secondary structure relative to N, as judged by the general decrease in intensity. Estimates for the secondary structure content of I are difficult because absorbance by urea precludes measurements below 207 nm, but the data suggest that a significant loss of helical structure occurs in the transition from N to I.

Fluorescence Spectra—The fluorescence spectrum for rMaspin reports primarily on the environment of its two tryptophan residues, Trp-171 and Trp-258 (25). The wavelength of maximum emission intensity, \( \lambda_{\text{max}} \), for native rMaspin is 343 nm and this shifts to 358 nm when rMaspin is denatured in 8 M urea (Fig. 5B). The latter value is as expected for solvent-exposed tryptophan side chains. The blue-shifted \( \lambda_{\text{max}} \) for native rMaspin indicates that one or both of the tryptophan side chains is at least partially buried in the nonpolar interior of native rMaspin.

The partial unfolding of N to I is associated primarily with small changes in fluorescence intensity and, possibly, a very modest red shift of \( \lambda_{\text{max}} \) (Fig. 5B). Thus, the environment of the tryptophan residues appears to be undergoing relatively modest changes in the transition from N to I. In contrast, \( \lambda_{\text{max}} \) undergoes a significant red shift in the transition from I to U, suggesting that buried tryptophan residues are exposed to solvent in this transition.

**DISCUSSION**

Chemical denaturation of rMaspin is a multistep process involving at least one intermediate, I, in addition to the native, N, and unfolded species, U. The spectral properties of U are consistent with a disordered polypeptide backbone and tryptophan residues that are well exposed to solvent. The denaturation profile is sensitive to protein concentration and this appears to result from self-association of I. Fluorescence data...
suggest that one or both of the tryptophan residues are buried in both N and I, but CD data indicate that I contains much less secondary structure than N. Much of the change in secondary structure appears to be loss of helix.

Detailed structural information is available for a small number of equilibrium unfolding intermediates in other proteins. The intermediates show native-like structure in a portion of the molecule while the rest of the protein is largely disordered (26). On this basis, the molecular model for native rMaspin can be used to generate hypotheses regarding possible structure in the I state. All serpins of known structure share a very similar overall fold (16). A model for the structure of maspin has been constructed on the basis of significant sequence identity with a serpin of known structure, ovalbumin (23, 27). The fold is dominated by the extensive β-sheet A, which bridges two major domains. One domain is rich in β structure and includes the RSL while the other domain consists of mixed α/β structure. Sequence alignment suggests that Trp-171 and Trp-258 of maspin correspond to Trp-184 and Trp-267 of ovalbumin. Both tryptophan residues are located in the domain that is rich in β structure.

The transition from N to I in rMaspin is accompanied by changes in tryptophan fluorescence intensity and an almost negligible red shift in λmax. This suggests that the tertiary structure in the β-rich domain is largely intact in I. In contrast, the far-UV CD data suggest a significant loss of helical structure. Most of the helical structure is located in a mixed α/β domain that is distal to the RSL. These observations lead to the hypothesis that I consists of a partially disordered α/β domain and a largely intact β-rich domain. Self-association of I may result from exposure of hydrophobic surfaces in the partially folded molecule or from insertion of the RSL into either β-sheet A or C, which appears to be the case for a number of other serpins (e.g., Refs. 14 and 15).

The fluorescence and far-UV CD spectra for the I state are very similar to those for equilibrium and kinetic intermediates in the folding of ovalbumin (28), although no self-association was observed in these studies. Lomas and co-workers (15) have recently illuminated the relationship between partial unfolding and self-association of α1-antitrypsin. More generally, the three-state chemical denaturation profile for rMaspin closely resembles those for other serpins such as α1-antitrypsin, antithrombin III, α1-antichymotrypsin, C1 inhibitor, and plasminogen activator inhibitor I (29–33). Ovalbumin is a clear exception in this regard and undergoes a more cooperative two-state unfolding reaction (29). For serpins that have undergone the S to R transition, chemical denaturation profiles are shifted toward higher denaturant concentrations and, in some cases, appear to reflect two-state transitions (29, 31).

Experimental and computational studies of multidomain proteins suggest that the cooperativity of denaturation reflects the properties of interfaces between domains (34–36). One hypothesis that follows from this analysis is that more cooperative transitions are observed in serpins that have more extensive interdomain interactions. This hypothesis may be testable with the rapidly growing data base of serpin structures (16).

In the case of rMaspin, the cooperativity of denaturation also reflects self-association of an intermediate, I. In fact, CD and fluorescence spectra for I (Fig. 5) are probably dominated by contributions from multimeric I species. Self-association of intermediate states may also explain the three-state chemical denaturation profiles observed for other serpins (29–33).

Does self-association of rMaspin explain its unusual dose-response behavior in studies of breast cancer cell motility and invasiveness? Native rMaspin does not self-associate but partially unfolded rMaspin undergoes self-association at concentrations where decreases in biological activity are observed. However, native rMaspin appears to be relatively stable at pH 7 and 37 °C (Fig. 4). These results suggest that, under the solution conditions examined in the present study, rMaspin does not self-associate to a significant extent. In principle, questions remain regarding the possible effect of other cell medium components on self-association. Indirect evidence indicates the real potential for such an effect: self-association of rMaspin appears to be induced by a chromogenic peptide substrate for plasmin.2

An alternative approach will be to examine the possibility that peptide bond cleavage in the RSL of rMaspin leads to self-association with intact rMaspin. rMaspin is very prone to partial proteolysis in the RSL (11, 23). Cultures of breast cancer cells accumulate proteinases (8), some of which may cleave the RSL of rMaspin. Little is known about the stability and self-association properties of RSL-cleaved rMaspin relative to intact rMaspin (11).

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