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Productive Folding of Human Neutrophil α-Defensins in Vitro without the Pro-peptide

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Human neutrophil α-defensins (HNPs) are small, Cys-rich, cationic antimicrobial proteins. Stored in the azurophilic granules of neutrophils, they are released during phagocytosis to kill ingested foreign microbes through disruption of their cytoplasmic membranes.[1−4] Recently, the three most abundant forms of human α-defensins, HNPs 1−3, have been implicated in suppressing HIV-1 infection in vivo, thereby exhibiting a potential therapeutic value in the treatment of AIDS.[5] HNPs are synthesized as inactive precursors in vivo and activated through proteolytic removal of their inhibitory N-terminal pro-peptide required for correct subcellular sorting and processing.[6−8] Folding of HNPs 1−3 in vitro without the pro-peptide has been reported to be extremely difficult,[9,10] which led to the hypothesis that the 45-residue anionic pro-peptide may assist proHNPs folding as an intramolecular chaperone interacting with the cationic C-terminal domain, a mechanism reminiscent of some bacterial serine proteases.[11,12] Here we show that HNPs without the pro-region can fold productively with yields over 80% in the presence of 2 M urea and 25% *N*,*N*-dimethylformamide (DMF). Our finding demonstrates an efficient protocol for the production of large quantities of highly pure human α-defensins and is broadly applicable in folding aggregation-prone, Cys-rich proteins of both synthetic and recombinant origin.

Folded HNPs 1−3 are known to adopt a three-stranded antiparallel β-sheet conformation constrained by three intramolecular disulfide bonds.[13,14] They are highly soluble in aqueous solution and are structurally stable even in 8 M urea. In contrast, the reduced form quantitatively aggregates in the absence of high concentrations of denaturants. Consequently, massive precipitation occurs as denaturing conditions are removed during the folding of HNPs in aqueous solution. Currently, the most effective protocol for folding HNPs without the pro-peptide utilizes the irreversible and nondiscriminating oxidant DMSO and typically results in 10% recovery.[9] Due to the exceptionally high stability of HNPs, we argue that preventing the aggregation of unfolded proteins by using sufficient amounts of denaturants, suitable organic cosolvents, or both facilitates productive thiol−disulfide exchanges in the presence of oxidized/reduced thiol pairs, thus leading to the formation of a stable native structure.

All folding experiments were conducted at pH 8.1 in a buffer solution containing 0.1 M $NaHCO_3$ and 3 mM reduced and 0.3 mM oxidized glutathione and differed only in the amounts of urea and/or DMF. We first examined the folding behavior of a 75-residue synthetic full-length proHNP1 at various urea concentrations. As shown in Figures 1A and 1B, the optimal concentration of urea is 2 M, yielding a 50% recovery after 8 h at room temperature. In the presence of 0.2 M urea, proHNP1, while fully soluble, also folds productively, although at a significantly slower rate. Notably, the folding of proHNP1 initially proceeds more efficiently in the presence of 4 or 6 M urea. However, the overall yield steadily declines after 4 h, presumably due to competing thiol−disulfide exchanges under more denaturing conditions.

Reduced HNP1 completely precipitates in 2 M urea, and serious aggregation occurs in 4 M urea. In contrast, the protein remains soluble in 6 M urea, resulting in a 50% conversion to the folded form after 4 h, although the yield also declines as the folding progresses. Significantly, the highest folding yield (80%) is achieved in a combination of 25% DMF and 2 M urea (Figures 1C and 1D). The presence of the organic cosolvent effectively eliminates aggregation of HNP1 and suppresses nonproductive thiol−disulfide exchanges seen with the folded protein at high concentrations of urea. It should be noted that less than 15% DMF in 2 M urea is not sufficient to prevent protein aggregation, whereas at higher than 30% DMF the folding yield progressively decreases as the percent organic solvent increases. Using the urea/DMF protocol, we have also folded HNP2 and HNP3 with similar efficiency (Supporting Information).

Several lines of evidence indicate that the highly pure synthetic HNPs are correctly folded and functional (Supporting Information). First, folding of fully reduced forms of HNPs results in a loss of six mass units, indicative of formation of three disulfide bridges. Second, complete proteolysis of HNPs by trypsin and chymotrypsin followed by one-step manual Edman degradation generates unique peptide fragments identified by mass spectrometry and allows for an unambiguous assignment of the native disulfide pairings, i.e., Cys^1−Cys^6, Cys^2−Cys^4, and Cys^3−Cys^5. Third, fluorescence spectroscopy studies of reduced and of folded HNPs show that the Trp fluorescence blue-shifts and is strongly quenched in the folded structure. This finding is consistent with the existence of a proximal disulfide and a strong H-bond (2.8 Å) between the side chain of a partially buried Trp residue and the main-chain carbonyl C═O group of a neighboring Tyr residue, as observed in the X-ray crystal structure of HNP3.[13] Lastly, HNPs 1−3 all demonstrate similar antimicrobial activity against both Gram-positive and -negative bacteria, with a minimum inhibitory concentration (MIC) of 11 μg/mL for *Escherichia coli* and of 33 μg/mL for *Streptomyces aureus*. It is worth pointing out that folded proHNP1 is inactive against the bacteria tested, consistent with the previously published observation that the pro-peptide acts as an intramolecular inhibitor through electrostatic interactions with the C-terminal mature form.[7] However, chemical cleavage of proHNP1 by cyanogen bromide yields an active HNP1 with the native S−S topology verified by disulfide mapping (data not shown).

An ideal solvent condition for productive protein folding, in principle, should be nondenaturing but effective in preventing the aggregation of unfolded molecules by solubilizing exposed hydrophobic residues. A delicate balance between the two opposing factors can be achieved for many proteins by the use of low concentrations of urea, which, as a strong H-bond donor and acceptor, preferentially stabilizes the denatured state of a protein through extensive H-bonding to its amide backbone structure.[15,16] In contrast, DMF more efficiently solvates hydrophobic compounds

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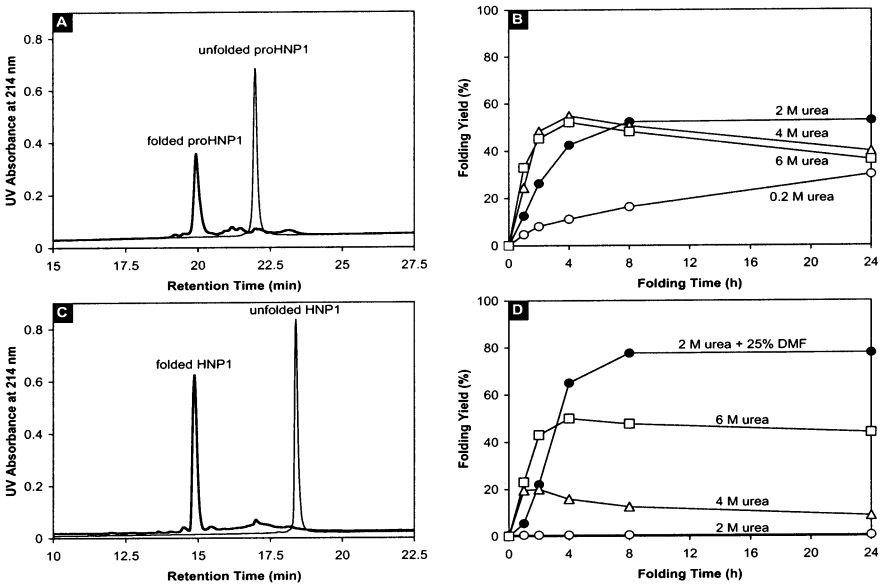

Figure 1. (A) ProHNP1 before (thin line) and after (thick line) 8-h folding at 0.5 mg/mL in the presence of 2 M urea. Reversed phase HPLC analysis was performed at 40 °C on a Vydac C18 column (4.6 × 150 mm) running a gradient of 5−65% acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min over 30 min. (B) Time dependence of folding yields for proHNP1 under different urea concentrations. No protein precipitation was observed. Samples were withdrawn at different time intervals for HPLC analysis, and the yields were calculated on the basis of the ratio of integrated areas of two peaks given by folded protein and fully reduced starting material, respectively. (C) HNP1 before (thin line) and after (thick line) 8-h folding at 0.25 mg/mL in the presence of 25% DMF and 2 M urea. HPLC analyses were carried out under the same conditions as described above. (D) Time dependence of folding yields for HNP1 under different solvent conditions. No protein precipitation was observed in the presence of 6 M urea or 2 M urea and 25% DMF.

such as heavily protected synthetic polypeptides, but is less effective than urea in competing for H-bonding due to N-alkylation.[16] Therefore, it is plausible that the use of DMF in combination with urea results in a more solubilizing but less denaturing solvent condition responsible for the productive folding of HNPs. If this mechanistic rationale is valid, we should expect the covalently conjoined pro-peptide to play a similar role in solubilizing the aggregation-prone C-terminal domain in proHNPs. Further, a significant sequence variation in the pro region ought to be tolerated in the folding of proHNPs as long as the solubilizing capacity of the pro-peptide is not seriously compromised. To test this hypothesis, we chemically synthesized a full-length mutant proHNP1 in which all the charged residues in the pro region were changed to residues of opposite charges, i.e., Arg/Lys to Asp, Glu/Asp to Lys. These mutations effectively disrupt the electrostatic interactions between the pro-peptide and the C-terminal domain in the mutant protein. The charge-switched pro-protein indeed folds productively at various urea concentrations (Supporting Information). This finding strongly suggests that the pro-peptide assists proHNPs folding by simply enhancing their solubility—a novel mechanism of intramolecular chaperoning without inter-domain interactions required in the folding of some bacterial serine proteases.[11,12] Further, unlike bacterial serine proteases, the presence of the pro-peptide in large excess, intermolecularly, does not impact the folding and aggregation of reduced HNPs in 2 M urea (data not shown).

Folding proteins in vitro is an essential step in the production of functional protein molecules expressed in *E. coli* as inclusion bodies or from chemically synthesized denatured forms. Unlike protein folding in vivo, which is aided by various accessory molecules or molecular chaperones and the regulated degradation of misfolded species, folding proteins in vitro, particularly those containing multiple disulfide bonds, can be challenging due to disulfide mispairing and irreversible aggregation. We have demonstrated that the use of DMF and urea strongly enhances folding efficiency of HNPs presumably through suppressing aggregate formation and nonproductive disulfide−thiol exchanges. The procedure is simple and cost-effective and can be broadly applied to the large-scale production of recombinant and synthetic proteins that are otherwise prone to aggregation in aqueous solution. Our finding sheds light on the role of the pro-peptide in the folding of HNPs and may also have implications on the molecular basis of protein misfolding and aggregation in various pathological cellular processes.

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Supporting Information Available: Chemical synthesis of HNPs and proHNP1 and characterizations by mass spectrometry, fluorescence spectroscopy, antimicrobial activity assay, and disulfide mapping. This material is available free of charge via the Internet at http://pubs.acs.org.

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