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The formation and mechanism of multimerization in a freeze-dried peptide

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Abstract

Atrial natriuretic peptide (ANP) is a peptide with 25 amino acid residues (hANP 4–28) and one intra-chain disulfide bond. We used the size-exclusion chromatography (SEC), sodium dodecyl sulfate in polyacrylamide gel electrophoresis (SDS-PAGE), and reversed-phase liquid chromatography with electrospray mass spectrometry (LC–MS) methods to examine the freeze-dried products of ANP to determine the types, sizes, and amounts of the multimer formation in different stability protocols (by varying the conditions with lyophilization cycles, excipients, storage temperatures, and times). Under the non-annealing lyophilization cycle or lyophilization with high concentration of bulking agent (mannitol), multimer formation increased with increasing storage times. Two kinds of multimers were observed: the major portion is reducible and the minor portion is non-reducible. The reducible multimers are disulfide-linked multimers as determined by LC–MS. The non-reducible multimer was mainly a dimer, possibly linked by a covalent bond between the side chain of tyrosine in one molecule and the dehydroalanine intermediate in another molecule, based on the evidences of the mass of the non-reducible dimer along with the elution position in SEC, and the change of the UV spectrum in the aromatic region. The analysis of degradants suggests that the mechanism start from an β-elimination of disulfide linkage to form a free thiolate ion (HS^-) and a dehydroalanine-type peptide intermediate. The HS^- then catalyzed ANP to form the disulfide-linked multimers. The dehydroalanine-type ANP intermediate then reacted with another ANP molecule to form a non-disulfide-linked dimer through reaction with the side chain of tyrosine. These results suggest that the source of multimer formation be initiated by phase transition (from amorphous to crystalline phase) either in the freeze-dried process or during storage. That phase change may induce a drastic change in pH and moisture to damage the peptide. The detailed mechanism and the kinetics of ANP multimerization are discussed. The formation of the multimers was diminished by using the thermal treatment (the annealing step) with a proper ratio of mannitol to ANP peptide in the lyophilization, and/or increase of the acetate buffer concentration in the formulation.

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1. Introduction

Aggregation of proteins and peptides is frequently the key concern in biopharmaceutical product development. A particular issue in Journal of Pharmaceutical Sciences had included papers (Chen et al., 1994; Shahrokh, et al., 1994; Weitzhandler et al., 1994; Yeo et al., 1994) from an ACS symposium on aggregation issues in pharmaceutical protein formulations, held at the national meeting in March 1994. This report presents the methods of identifying and quantifying disulfide-linked multimers of a 25-amino-acid peptide observed in solid phase and proposes a mechanism that will explain the observations.

Although many other articles had used the term of 'aggregation' for high number of protein molecules joined together by either covalent or non-covalent bonds, we use 'multimerization' because we distinctly observed dimer, trimer, etc. of a peptide that is joined by only disulfide bonds.

AURICULIN®anaritide (human ANP 4–28) is manufactured by Scios Inc. for clinical studies to treat acute renal failure (Allgren et al., 1997). Its pharmacological functions include natriuresis, diuresis, and hypotension (Brenner et al., 1990). The ANP described in this report has 25 amino-acid residues with an intra-disulfide bond and has a molecular weight of 2724.1 Da (Fig. 1). The clinical study (Allgren et al., 1997) revealed that a 25–30 mg dose of ANP may be required. It would be preferred to package the total required dose in a single vial (earlier clinical trials used a 5 mg/vial product). To obtain the single-vial package, we need to freeze dry a higher concentration of ANP (e.g. 2.5–5 mg/ml) with proper formulation excipients and lyophilization process. To define optimal conditions, we varied the amount of bulking agent (e.g. mannitol), buffer (acetic acid), and lyophilization cycles, as well as the concentration of ANP. Samples from the various preparations were analyzed by several analytical methods. These changes in ANP compounding formulation and lyophilization cycle were relatively minor, since the composition, buffer pH, freezing temperature, and drying pressure were kept similar to each other.

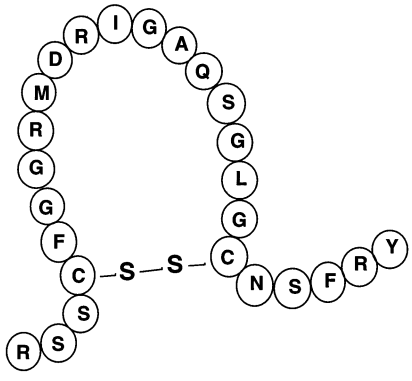

Fig. 1. Amino acid structure of ANP.

Often, in the pharmaceutical industry, if an existing product is progressing in clinical studies, formulation change is strictly constrained to avoid another lengthy clinical trial just for safety reasons. The most drastic change here is the increased ANP concentration from 1 mg/ml in the current process to 2.5 or 3.6 mg/ml in the proposed compounding formulation. Because of the increased ANP concentration, aggregation or multimerization is the primary concern.

Therefore, a sensitive and quantitative method for measuring the multimers is needed. Reversed-phase HPLC (RPLC) is good for separating ANP variants, but not for separating large multimers (low recovery). Ion-exchange chromatography (IEC) is good for separating ANP charge variants, but not for discriminating multimer forms (broad peak). Sodium dodecyl sulfate in polyacrylamide gel electrophoresis (SDS-PAGE) is good for separating ANP multimers, but is not accurate for quantitation. Size-exclusion chromatography (SEC) is good for separating ANP multimers and good for quantitating.

Therefore, we focused on SEC method development. In the following, we will show how to use the SEC method to optimize the lyophilization cycle and describe the mechanism of the multimer formation.

2. Materials and methods

2.1. Materials

2.1.1. Equipment

HPLC: HP1090 or 1100 equipped with a diode array detector and ChemStation computer from Hewlett Packard (Waldbronn, Germany).

Column: Toso Haas (Montgomeryville, PA) TSKgel G2000SWXL (7.8 mm × 30 cm, 5 μ, 125 A).

2.1.2. Materials

ANP product, 25 mg/vial, was prepared by Scios Inc. (Mountain View, CA). The ANP drug substance was chemically synthesized by Bachem (Torrance, CA). The compounding formulation in each vial contains either 7 ml of 3.6 mg/ml ANP with 5 mM acetic acid or 10 ml of 2.5 mg/ml ANP with either 5 or 15 mM acetic acid, and with different amounts of mannitol (e.g. 1, 3.6, 5, and 10% mannitol, w/w). These samples were freeze-dried using the cycles with or without an annealing step (Fig. 3), and with the vacuum set at 100 mTorrs (μmHg). Samples were stored at different temperatures and analyzed at various time intervals.

The molecular weight standards were prepared by mixing three synthetic peptides with molecular weights of 826, 1593, and 3894 Da (Synchrom, Inc., IN). Dithiothreitol (DTT) was purchased from EM Science (Princeton, NJ). The 10–20% pre-cast gradient gels were obtained from Integrated Separation Systems (San Diego, CA)

2.2. Methods

2.2.1. SEC

The analyses were performed by isocratic elution using a mobile phase containing 70% of 10 mM TFA and 100 mM NaCl in H_2O and 30% acetonitrile (v/v) at a flow rate of 0.5 ml/min, and at ambient temperature for column and sample compartments. Lyophilized samples were reconstituted with water or 5% dextrose (D5W) to obtain ANP concentration at either 3.6 or 1 mg/ml ANP concentrations (as specified in the report) and an aliquot of 50 μl or otherwise specified was injected. Detection was performed at either 214 or 280 nm, depending on the amount of sample load.

2.2.2. On-line photodiode array

Each spectrum was recorded from 200 to 400 nm at 0.3-s intervals over an entire SEC elution. Sample spectra between 245 and 335 nm were abstracted and normalized to the highest intensity in this wavelength region for comparisons.

2.2.3. SDS-PAGE

A Novex Mini-Cell (Novel Experimental Technology, San Diego, CA) with MiniPlus TC (tricine compatible) SepraGel, 10–20% gradient with 12 wells, 1 mm thickness (Integrated Separation System, San Diego, CA) were used. The non-reducing buffer contained: 900 mM Tris–HCl, pH 8.4; 30% glycerol; 2% SDS; 0.01% EDTA; 0.005% phenol red. The reducing buffer was prepared by the addition of 320-mM DTT to the non-reducing buffer. ANP samples were reduced by one of the following protocols: by adding the reducing buffer or the reducing buffer containing 6 M urea for more than 4 h at room temperature, or by heating the samples at 100°C for 5 min in the reducing buffer or in the reducing buffer containing 6 M urea. Seven or 14 μg of samples, either intact or reduced, were loaded per well. The electrophoresis was performed using the Tricine Running Buffer, pH 8.3 from a 1:10 dilution of the 10X Tris/Tricine/SDS Premixed Buffer (Bio-Rad, Richmond, CA) at a constant current of 55 mA for 1 h. Molecular weight markers (Mark 12 Wide Range Protein Standard, Novel Experimental Technology, San Diego, CA) was also run alongside with ANP samples. Following electrophoresis, gels were stained using a Daiichi silver stain kit (Integrated Separation Systems, Natick, MA) according to the instruction for high-contrast staining. For destaining, gels were immersed in 30% ethanol, 5% glycerol, 65% water with repeated changes of this solution until the desired background was obtained (stopped by 1% acetic acid solution).

2.2.4. LC–MS

The collected fractions from SEC were directly injected into reversed-phase HPLC on-line with MS. A HP 1090 HPLC system and a Vydac C-4 column (2.1 × 250 mm) were used for the analyses. Solvent A consisted of 35-mM acetic acid in water and solvent B consisted of 35-mM acetic acid in methanol. The elution condition started with a hold at 15% B for 3 min and then ramp to 80% B over 20 min followed by holding at 80% B for 12 min. The flow rate was 0.2 ml/min and the column was kept at ambient temperature. A Finnigan MAT SSQ MS (San Jose, CA) equipped with an electrospray ion source and a single quadrupole analyzer was used for the MS analyses. The quadrupole was set to scan from m/z 500 to 2400 with scan duration of 3 s. The mass spectrometer was set with following parameters: electrospray voltage 4.5 kV, capillary temperature 230°C, and sheath gas pressure at 80 psi.

2.2.5. DSC

2.2.5.1. Instrument. Perkin–Elmer DSC 7, differential scanning calorimeter, with graphics plotter 2 and IBM computer (Sunnyvale, CA).

For a liquid sample, approximately 30 μg of sample was placed into a DSC pan. The sample was loaded at 25°C. The sample was then cooled to −40°C at 1°C/min. After equilibrated at −45°C, the sample was then heated to 25°C at 1°C/min. For a solid sample, approximately 0.5–3 mg of sample was placed into a DSC pan. The sample was heated from 25°C to a set temperature (170 or 40°C) at 10°C/min.

2.2.6. Disulfide reduction

An aliquot of 50 mM DTT in 50 mM Tris pH 8 with 2 mM $CaCl_2$ was mixed with an aliquot of 1 mg/ml ANP solution (1:2 v/v) and the mixture was allowed to sit at room temperature for at least 2 h prior to SEC analyses. Two types of reduced samples, with and without the additional 6 M urea, were prepared for use in SEC and SDS-PAGE.

3. Results

3.1. SEC of small peptides

Small peptides tend to interact with SEC stationary phases, and therefore lose the sieving effect of this method (Kato et al., 1980; Bennett et al., 1983). We evaluated various salts, detergents, pH, and organic solvents, and found that at least 30% acetonitrile is needed to diminish the interaction of ANP with various SEC stationary phases. We have observed similar approaches in other separations of small peptides by SEC columns (Swergold and Rubin, 1983; Suda et al., 1984; Irvine and Shaw, 1986). We also found that low pH (pH below 4) is important for ANP recovery. Thus, an efficient separation of ANP multimers was developed by using 30% acetonitrile with 0.1 M sodium chloride and 0.1% TFA in the mobile phase shown in Fig. 2. As the figure shows, we estimated the ANP molecular weight by comparing its elution time with the elution time of the three synthetic peptide standards. The estimated molecular weight of ANP was 1824 Da, which was 900 Da less than the theoretical value. For such a small peptide, that discrepancy in SEC is common (Swergold and Rubin, 1983; Suda et al., 1984; Irvine and Shaw, 1986).

3.2. Effect of annealing

Fig. 3 shows two types of lyophilization cycles — annealing and non-annealing cycles. The difference between these two cycles is a thermal treatment process (annealing) that increases the freeze temperature from −50 to −10°C and then decreases it to −50°C again.

Fig. 4 shows that the incorporation of the annealing cycle reduced the tendency to form multimers under accelerated storage conditions. The amount of multimers were 17% (Fig. 4A, without annealing cycle) and 0.46% (Fig. 4B, with annealing cycle). The other preparation and storage conditions were kept equal in this study (e.g. both contained 1% mannitol and stored at 40°C for 2 months). Also, to rule out factors that might affect the multimerization, we examined the moisture content, the cake appearance, and the final

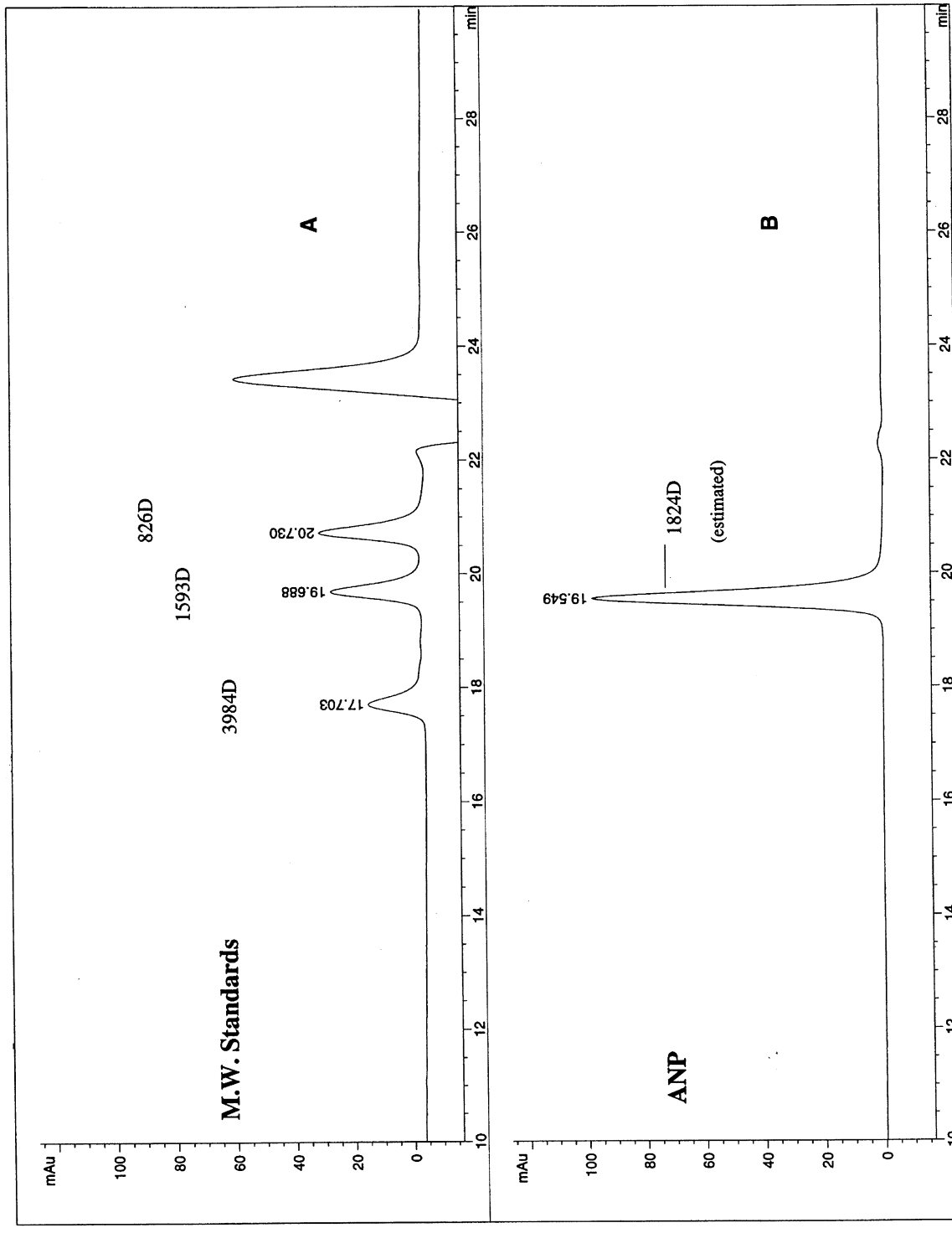

Fig. 2. Comparison of the SEC elution profiles of (A) molecular weight standard of three peptides, and (B) ANP peptide. An aliquot of 50 μl was analyzed by SEC.

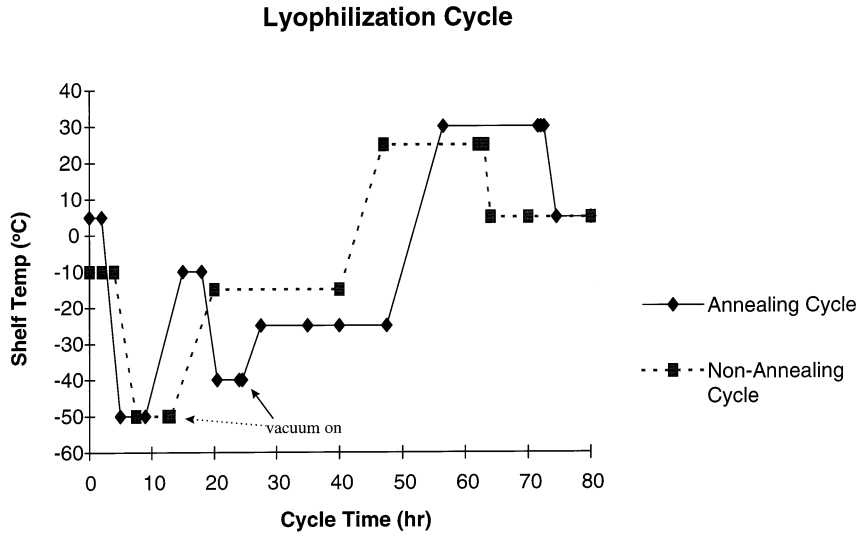

Fig. 3. Lyophilization of ANP under the annealing (◆), and the non-annealing cycle (■) as indicated by the freezing shelf temperature and drying vacuum.

pH for both ANP products (with and without annealing cycles) and showed comparable results. Both of them had low moisture content (1.2% w/w), good appearing cake, and similar pH (pH 5.5, after reconstitution with water). Considering this observation in Fig. 4, we added the annealing step in the lyophilization cycle and then examined the effect of mannitol concentration.

3.3. Effect of mannitol

We found that the sample with 1% mannitol had the least amount of multimers (0.30% aggregates), as compared to the samples with 3.6% mannitol (0.40% aggregates), 5% mannitol (0.48% aggregates), and 10% mannitol (1.92% aggregates). The preparation and storage conditions were kept equal in this study (e.g. used the same annealing cycle with the ANP concentration at 3.6 mg/ml and stored the samples at 40°C for 2 months).

The non-annealing cycle or the annealing cycle with the high ratio of mannitol to ANP lead to damage the ANP product even in the early shelf life. These two parameters are critical in developing a stable ANP. Subsequently, by controlling these two factors (choosing the annealing cycle with the ratio of 1% mannitol to 3.6 mg/ml ANP), we observed a stable ANP product as shown in the following.

3.4. Effect of storage temperature and time

At 2-months we observed only dimer formation (no multimer) under accelerated temperature storage; 0.19% at 5°C, 0.29% at 25°C, and 0.57% at 40°C. Under the sub-ambient temperature storage (5°C), we observed only dimer formation, no multimer; 0.12% at initial, 0.2% at 2 months, and 0.37% at 1 year.

When using a non-optimized condition — for example, using the non-annealing cycle or the annealing cycle with the ratio of 10% mannitol to 3.6 mg/ml ANP, the amount of multimers increased to 20% at 25°C storage for 3 months and to 100% at 40°C for 3 months. Also at this stage, some multimers became insoluble.

3.5. Analysis by SEC and SDS-PAGE

We were intrigued by the formation of the large amounts of multimers, particularly in the non-annealing lyophilization cycle after short-term storage. This phenomenon prompted us to investigate the nature of the multimers. First, we reduced the multimers by DTT and examined the reduced

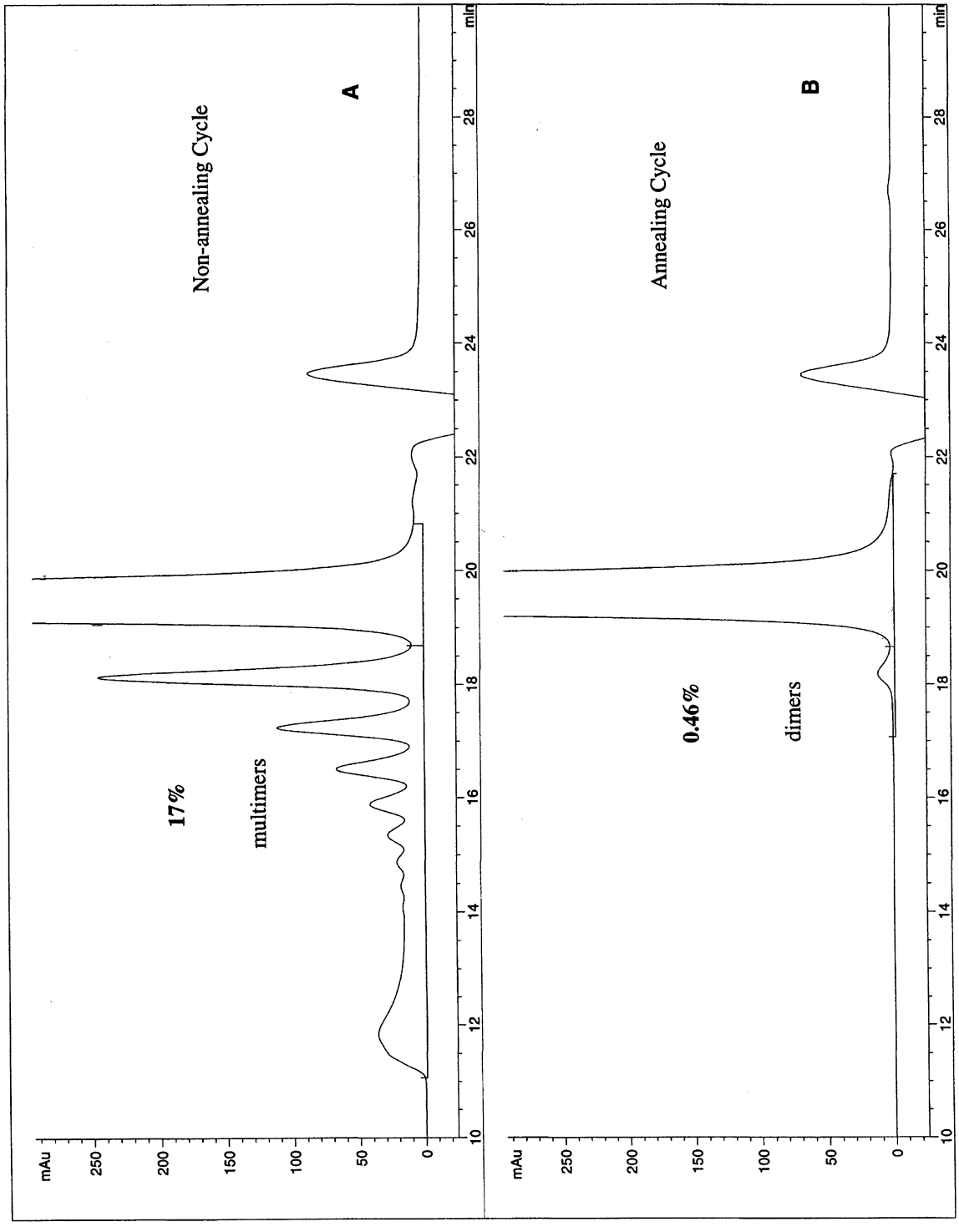

Fig. 4. Comparisons of the SEC elution profiles of ANP produced under different lyophilization cycles. (A) without annealing step, and (B) with annealing step.

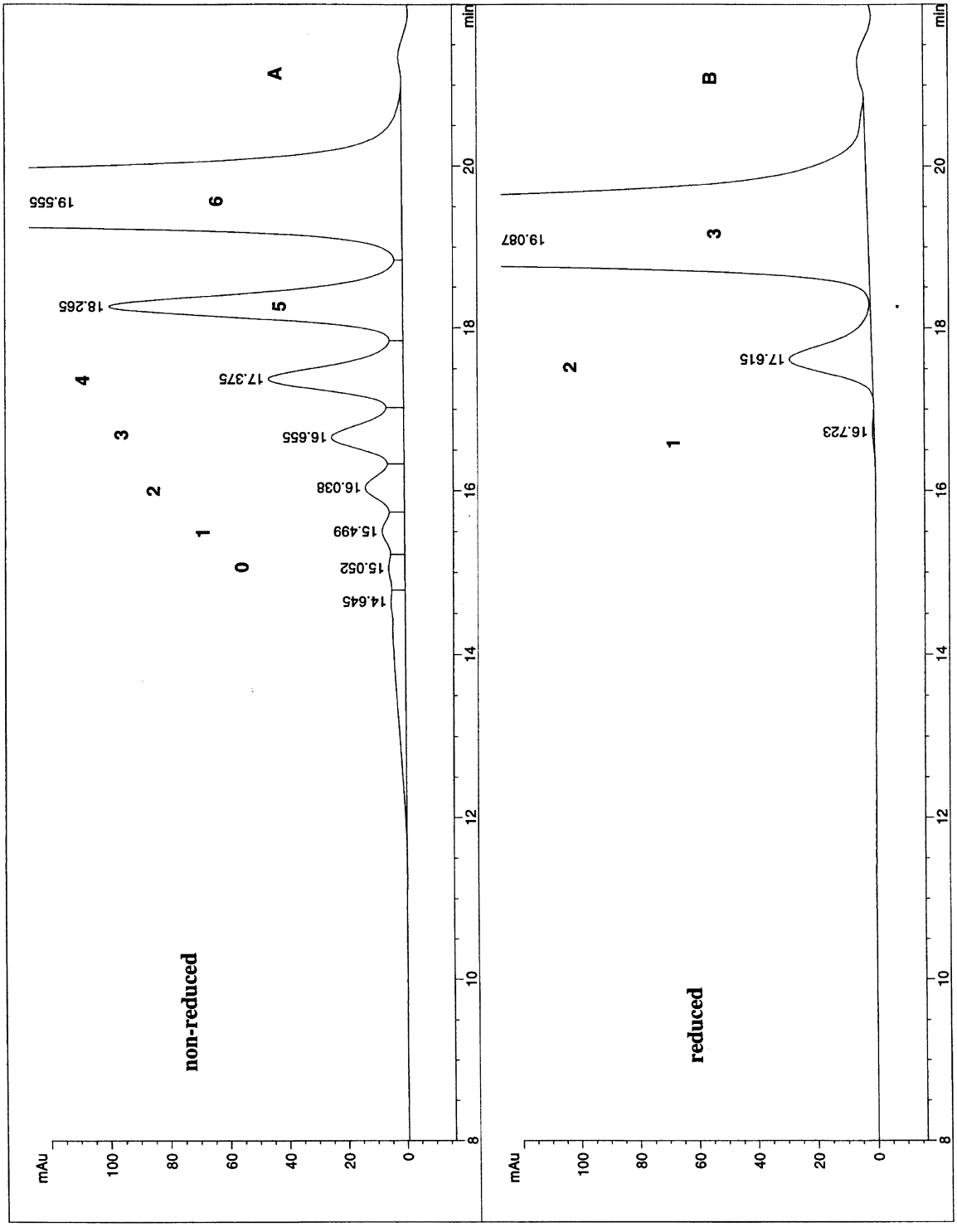

Fig. 5. Comparison of the SEC elution profiles of ANP products treated (A) with or (B) without DTT. Each peak was labeled with its elution time along with the assigned peak number (from 0 to 6 in A and from 1 to 3 in B).

multimers along with the intact multimers (no DTT treatment) by SEC, SDS-PAGE, and LC–MS as shown in Figs. 5 and 6 and in Table 1, respectively.

As shown in Fig. 5A, the multimers are eluted like dimer, trimer, tetramer, pentamer, hexamer, and so on to very large aggregates (up to the excluded volume of SEC). Also, most of these aggregates disappeared when reduced with DTT as shown in Fig. 5B. Based on peak areas (Fig. 5B), most of these reduced (or disappeared) portions eluted in the monomer position. These results suggest that most of these multimers are disulfide-linked. As shown in Fig. 1, ANP has only one disulfide bond. Reduced ANP monomer can be anticipated to be a more extended (or larger) molecule than the non-reduced ANP monomer. This explains why the reduced monomer (peak 3) in Fig. 5B eluted slightly earlier than the non-reduced monomer (peak 6) in Fig. 5A (eluted like 1.5mer). Fig. 5B shows the low percentage of non-reducible dimers (peak 2). Also, this non-reducible dimer was bigger than the disulfide-linked dimer (eluted like 2.5mer). We will discuss this phenomenon in the discussion section.

Fig. 6 also shows the intact aggregates and their reduced forms as represented by silver-stain SDS-

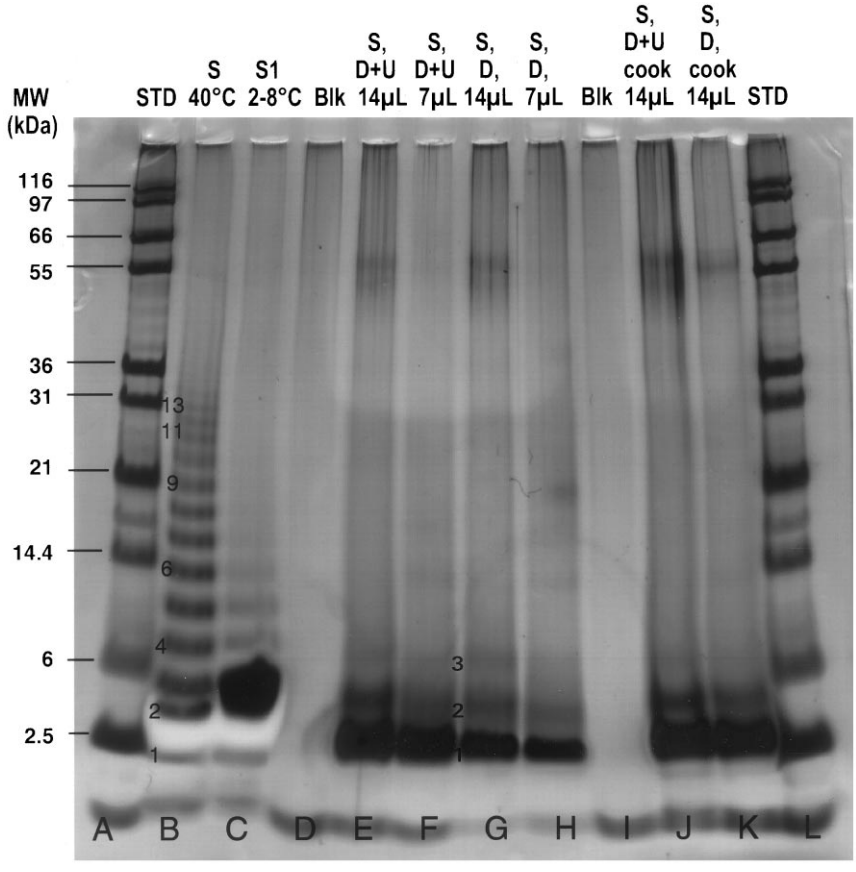

Fig. 6. Comparison of the SDS-PAGE profiles of the same ANP products in Fig. 8 treated with or without DTT. Lanes A & L: molecular weight standards. Lane B: ANP sample, 10-µl load (non-reduced and stored at 40°C for 2 months). Lane C: ANP sample, 10-µl load (non-reduced and stored at 2–8°C for 2 months). Lanes D and I: blank lanes (no sample). Lanes E and F: the same sample in lane B but treated with DTT (25 mM) and urea (6 M); a 14-µl load for Lane E and a 7-µl load for Lane F. Lanes G and H: the same sample in Lane B but treated with DTT only (25 mM); a 14-µl load for Lane H and a 7-µl load for Lane G. Lanes J and L are the same samples as in Lane H and G, respectively, except heated at 100°C for 5 min.

Table 1
MS results of the SEC fractions

Fractions	Degree of multimerization	Expected masses[a]	Observed masses
A. No treatment[b]			
0	Heptamer	19 068.7	19 068.9
1	Hexamer	16 344.6	16 344.1
2	Pentamer	13 620.5	13 621
3	Tetramer	10 896.4	10 897
4	Trimer[c]	8172.3	8172.7
5	Dimer[c]	5448.2	5448.5
6	Monomer	2724.1	2724.3
B. Treated with DTT[d]			
1	Trimer (non-reducible)	8162.7	Undetermined
2	Dimer (non-reducible)	5418.5	5419
3	Reduced monomer	2726.3	2726.5

[a] Expected masses were calculated from average molecular weights.
[b] ANP sample was separated by SEC and the separated fractions were collected as shown in Fig. 5A.
[c] Fractions analyzed by LC–MS yielded two peaks with the same molecular mass.
[d] ANP sample was separated by SEC, and the separated fractions were collected as shown in Fig. 5B.

PAGE. The non-reduced sample (lane B in Fig. 7) shows that the aggregates can form up to the 13th mer (based on the number of bands in lane B), and the reduced sample (lane G) shows that these aggregates are reduced mainly to the monomer with a small amount of non-reducible dimer and a trace amount of non-reducible trimer.

Other lanes of samples in Fig. 6 (lanes E, F, H, J, and K) were treated slightly differently (e.g. by adding urea, heating for 5 min at 100°C, and changing amounts of load) to confirm the completeness of reduction as shown in lane G. All these results suggested again that most of the multimers are disulfide-linked multimers and that the small amount of non-reducible dimer and the trace amounts of non-reducible trimer in lane G are likely to be covalently linked by other types of bonds.

3.6. Analysis by LC–MS

The intact aggregates and their reduced forms from SEC in Fig. 5A and B were collected and then analyzed by reversed-phase HPLC on-line with a mass spectrometer (LC–MS). At first, we had difficulty with the LC–MS method, which yielded very low recovery and ionization efficiency when using the TFA buffer with an acetonitrile gradient in detecting those multimers (data not shown). Later, we developed a LC–MS method by using acetic acid buffer with a methanol gradient and obtained a very high MS efficiency. The masses are shown in Table 1.

In the untreated sample, the molecular weight of the SEC fractions (indicated by the collection numbers 6–0 in Fig. 5A) are consistent with the disulfide-linked monomer, dimer, trimer, tetramer, up to the heptamer as identified by MS and shown in Table 1A.

In the reduced (or treated with DTT) sample, the molecular weight of the major fraction of SEC (eluted like 1.5mer and indicated by the peak 3 in Fig. 5B) is consistent with the reduced monomer. Because of the addition of two hydrogens in reduction, the mass of the reduced monomer was two Da more than the untreated monomer as compared the fraction 3 of Table 1B with the fraction 6 of Table 1A. The molecular weight of the minor fraction of SEC (eluted like 2.5mer and indicated by the peak 2 in Fig. 5B) is also illustrated by MS and shown in Table 1B. The mass of this minor fraction (the peak 2 in Fig. 5B) is 30 Da less than the untreated dimer, from the comparison of the fraction 2 of Table 1B with the fraction 5 of Table 1A. This minor fraction is called a non-reducible dimer because its molecular

mass is close to the untreated dimer even after the DTT treatment.

3.7. Further analysis by on-line UV photodiode array

Fig. 7 shows the overlaid UV spectra of these five different ANP species eluting in different SEC positions, indicated as disulfide-linked monomer, dimer, trimer, the reduced monomer, and the non-reducible dimer, respectively (referred to as peaks 6, 5, and 4 in Fig. 5A and peaks 3 and 2 in Fig. 5B, respectively). As shown in Fig. 7, the absorption in the disulfide-bond region (approximate 245 nm) was higher for the disulfide-linked dimer and trimer, and was lower for the reduced monomer as compared to the intact (or disulfide-linked) monomer.

These results are consistent with more disulfide bonds in the disulfide-linked dimer and trimer, and no disulfide bond in the reduced monomer. The absorption in the aromatic region (λ maximum at 277 nm) of these disulfide-linked dimer, trimer, and the reduced monomer were not different from the intact monomer. These spectra were normalized to the highest intensity in the wave-

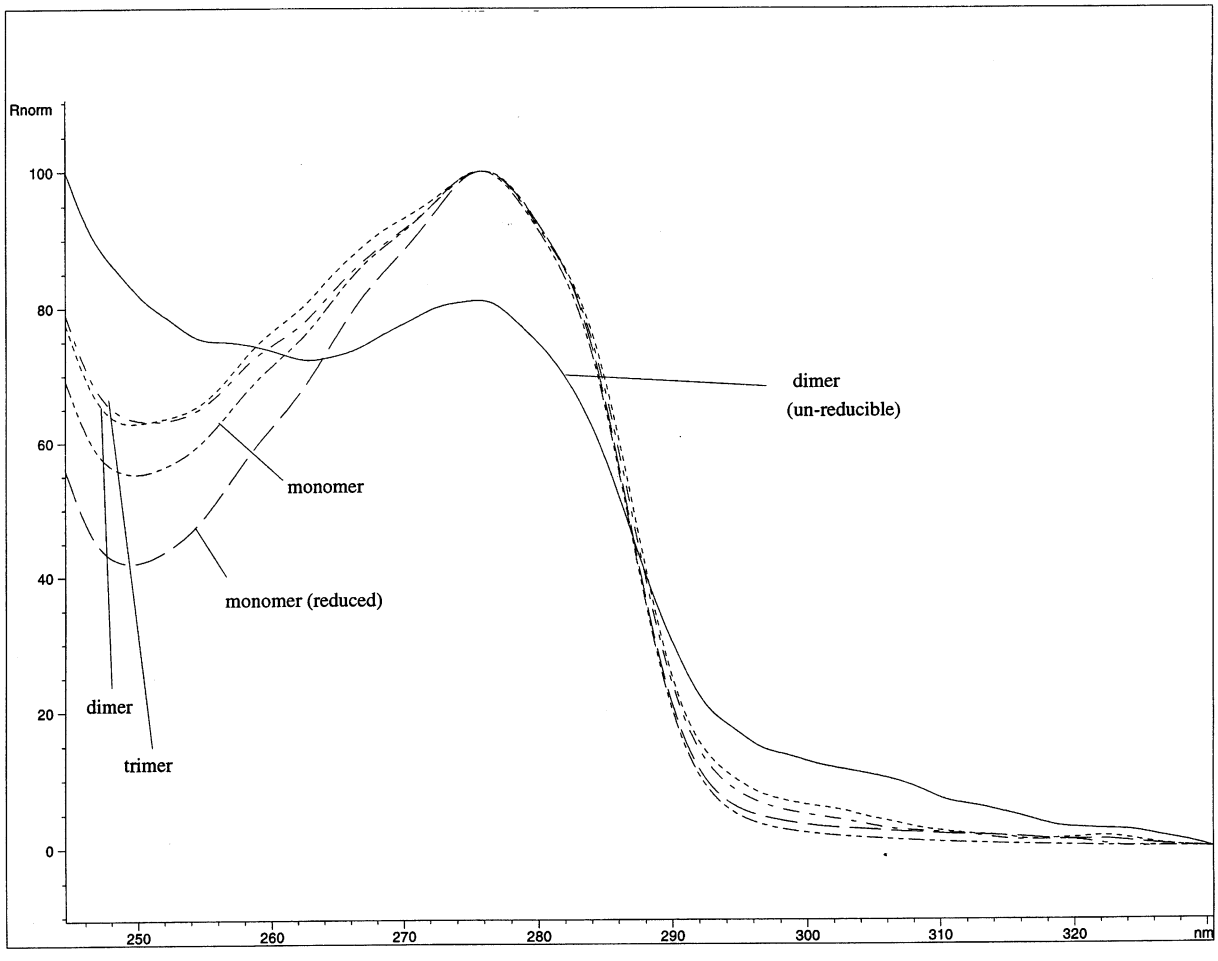

Fig. 7. Overlaid UV spectra of five different ANP species eluting in different SEC positions, indicated as the disulfide-linked monomer, dimer, trimer (referred to as peaks 6, 5, 4 in Fig. 5A, respectively), reduced monomer, and non-reducible dimer (referred to as peaks 3 and 2 in Fig. 5B, respectively). All spectra were recorded from 245 to 335 nm, and normalized to the highest intensity in this wavelength region.

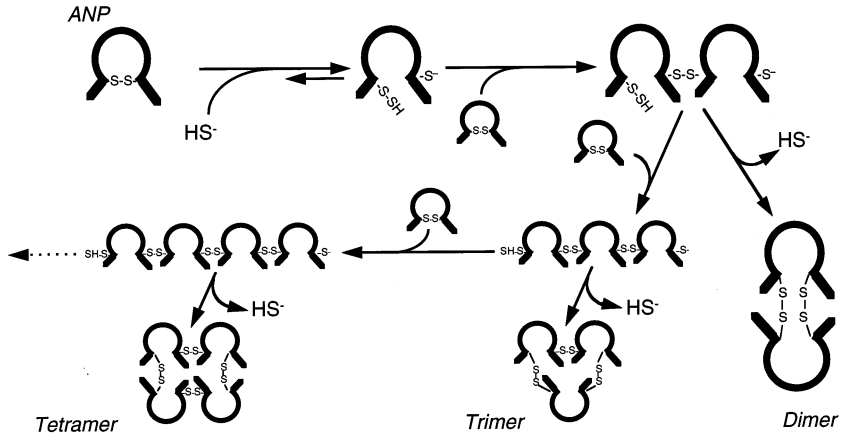

Fig. 8. The mechanism for the formation of the disulfide-linked ANP multimers.

length region between 245 and 335 nm. The λ maximum was still at 277 nm for those disulfide-linked monomer, dimer, trimer, and the reduced monomer indicated that the aromatic ring of tyrosine was still intact.

However, the non-reducible dimer had a quite different UV spectrum (the maximum absorption shifted from the 277 nm to the low wavelength region, 245 nm or less) as shown in Fig. 7. A bond other than a disulfide bond may link this non-reducible dimer together.

4. Discussion

4.1. The identity of the reducible and non-reducible multimer

We observed two kinds of multimers, one reducible and the other non-reducible, as shown by SDS-PAGE in Fig. 6 and by SEC in Fig. 5. The reducible multimers are disulfide-linked multimers based on their molecular masses and their reducible nature by dithiothreitol. The formation of the disulfide-linked multimers can be easily realized as shown in Fig. 8, which formed first through dimer, then trimer, tetramer, and so on to the large multimers.

The non-reducible dimer was first observed because it could not be reduced by dithiothreitol. MS showed that the mass of the non-reducible dimer was 30 Da less than the disulfide-linked dimer, which suggested that a bond other than disulfide bond might link between the dimer. To identify the linkage of this non-reducible dimer requires the correlation of our results with the observations by others. First, the non-reducible dimer in SEC eluted like a 2.5 mer after DTT reduction, which indicating that in this dimer, its disulfide bonds were reduced but still linked together by another covalent bond. Therefore, it was bigger than the dimer that was held together by two disulfide bonds. This is the same phenomenon as observed with the reduced monomer that behaves bigger than the disulfide-linked monomer in SEC and SDS-PAGE.

Second, this covalent-linked and non-reducible dimer was 30 Da less than the disulfide-linked dimer, fitting into the formation of a covalent bond but not a disulfide bond as proposed by others (Costantino et al., 1994a,b; Schwendeman et al., 1995) and shown in Fig. 9. Costantino et al. also observed the reducible and non-reducible aggregates in other proteins with high pH formulation (Costantino et al., 1994a). The non-reducible dimer was covalently linked by the side chain of lysine in one molecule and the dehydroalanine intermediate in another molecule as reported by them.

Third, ANP has no lysine or histidine residues. Therefore, only the side chain of tyrosine or the N-terminal amino group is reactive. The non-reducible dimer had a different UV spectrum in the aromatic region (tyrosine region). The change in

tyrosine spectrum indicated strongly that the linkage was through the side chain of tyrosine, not the N-terminal amino group.

4.2. The mechanism of multimer formation

Previously in several reports, Klibanov and Langer's groups (Liu et al., 1991; Costantino et al., 1994a,b; Schwendeman et al., 1995) had observed similar multimers, the reducible and non-reducible multimers. They proposed that multimerization was initiated by β-elimination to form a free thiolate ion (HS^-) and a dehydroalanine-like intermediate. The disulfide-linked multimers were formed through the catalysis of the HS^-. The non-reducible dimer was covalently linked by the side chain of lysine in one molecule and the dehydroalanine intermediate in another molecule. We postulate that the HS^- then catalyzes ANP to form the disulfide-linked multimers (Fig. 8), and the dehydroalanine-like ANP intermediate forms a non-disulfide linked dimer through reaction with the side chain of tyrosine as shown (Fig. 9).

As shown in Fig. 9, the non-disulfide linked dimer was formed through a dehydroalanine form of thiocysteine-ANP intermediate by β-elimination. This intermediate can quickly convert to a dehydroalanine form of cysteine-ANP intermediate by releasing a sulfur atom and then form either the carbon–nitrogen linked dimer through the N-terminus of ANP (ANP has no lysine or histidine residues) or the carbon–carbon linked dimer through the side chain of tyrosine. The carbon–nitrogen (C–N) or carbon–carbon (C–C) linked dimer cannot be reduced by DTT and should have a molecular weight of 30 Da less than the disulfide-linked dimer. That minus 30 dalton value was obtained through the loss of a sulfur (minus 32 Da) with the addition of two hydrogens after DTT reduction (plus 2 Da). The non-reducible dimer, observed in SEC (Fig. 5B) and SDS-PAGE (Fig. 6 in lane G), had a molecular weight of 30 less than the disulfide dimer as shown in Table 1B. These results support the postulated mechanism as shown in Fig. 9.

4.3. The source of multimerization

The purpose of incorporating an annealing step is to reduce non-frozen or super-cool molecules by promoting crystal formation of the compo-

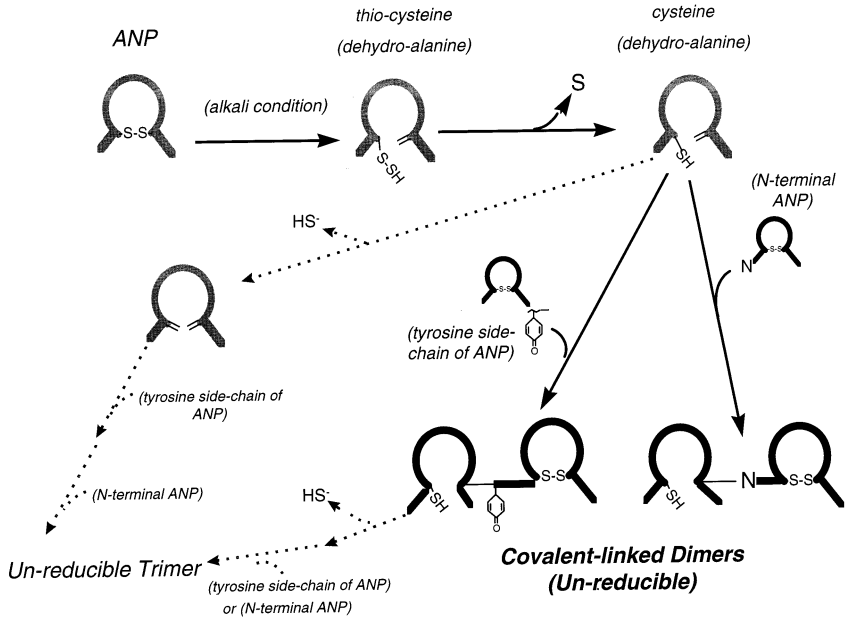

Fig. 9. The mechanism for the formation of the non-disulfide-linked ANP dimer and trimer.

nents and/or growth of ice crystals; both of these results are thought to facilitate ice sublimation resulting in more efficient drying of the product. Mannitol is usually used as a bulking agent for lyophilization with protein drugs and in some cases is not considered as a lyophilization protectant (Arakawa and Timasheff, 1982; Griebenow and Klibanov, 1995). However, others have reported that mannitol at lower concentration (1% or less) protected proteins from aggregation by forming an amorphous structure; at higher concentration, mannitol enhanced protein aggregation by forming a crystalline structure (Izutsu et al., 1993).

In our cases, without the annealing cycle, some fraction of the mannitol may remain in a metastable glass. During storage, this glassy state mannitol is crystallized and this phase change during storage may result in the drastic change in pH and moisture level for the remaining amorphous phase containing the peptide. These changes may cause ANP multimerization, as observed in Fig. 4A. By employing an annealing step, the mannitol is crystallized more completely and the phase transition during storage in the dried solid is avoided. However, as the ratio of mannitol to peptide increased, just an annealing step may not crystallize mannitol completely. That may explain why we observed more peptide multimerization when the ratio of mannitol to peptide increased.

From the evaluation of the lyophilized cake's quality in different mannitol-to-peptide ratios with or without an annealing step by differential scanning calorimetry, we could not observe any quantitative differences of these lyophilized cakes (all cakes seemed to be crystallized). At this stage we did not have a more quantitative tool such as X-ray diffraction spectrometry to discriminate the extent of crystallization. However, it may only take trace amount of catalyst (e.g. $HS-$) to trigger multimer formation. For this case, the trace amount difference in crystallization would not be detected by X-ray diffraction spectrometry either.

Buffer pH and protein concentration can be changed dramatically during phase transition in lyophilization or during storage (Kiovsky and Pincock, 1966; Brands and Nordin, 1970; Franks, 1991). The pI of ANP is more than 12, and the formulation buffer pH (5 mM acetic acid) for ANP is 4. During phase transition, the buffer pH can increase dramatically because of the high pI of ANP. In this circumstance, the formation of the $HS-$ and the dehydroalanine intermediate are likely. The formation of $HS-$ has also been observed by others when proteins were lyophilized in alkali buffer and the lyophilized product was stored at elevated moisture content, and has been categorized as moisture-induced aggregation (Costantino et al., 1994a,b). This phenomenon is similar to the formation of the $HS-$ and the dehydroalanine intermediate in alkali solution as shown by others as well (Cecil and McPhee, 1959; Catsimpoolas and Wood, 1964, 1966; Torchinsky, 1981).

A lower pH or a stronger buffer concentration may also prevent the buffer pH from becoming high when the phase transition occurs during storage and therefore should diminish $HS-$ formation and protein aggregation (Costantino et al., 1994a,b). We observed that the ANP dimer decreased from 0.55% to non-detectable after 3 months at 40°C storage when we increased the acetic acid buffer from 5 to 15 mM during lyophilization. Also, when we increased the moisture content to 4% with the storage temperature at 40°C for 1 week, we observed a significant increase in multimer formation in the 5 mM acetic acid buffer preparation (multimers increased to 2% level), but not in the 15 mM acetic acid buffer preparation with similar moisture content and storage conditions. This result suggested that the higher buffer concentration may prevent a pH during lyophilization or storage.

4.4. The kinetics of ANP multimerization

We also observed another trend: the disulfide-linked multimers increased significantly with increasing storage time, but the non-reducible dimer and trimer did not. This phenomenon could be explained by the formation of $HS-$. The $HS-$, even in trace amount, was a catalyst, which triggered the formation of disulfide-linked multimers continuously as shown in Fig. 8. However, the

non-reducible dimer and trimer were formed through the dehydroalanine-like ANP intermediate. This intermediate was a reactant. Once it reacted with ANP to form the non-reducible dimer and trimer, it was consumed. So, we believe that a small amount of HS− and dehydroalanine-like ANP intermediate were formed during the phase changes in lyophilization storage. Depending on the storage temperature, moisture, and time, the small amount of HS− (catalyst) could then trigger ANP to form the large amounts of disulfide-linked multimers. However, the small amount of dehydroalanine-like ANP (substrate) could only form a small amount of the non-reducible dimer and trimer through reaction with ANP, as shown in Fig. 5B. This catalytic phenomenon may explain that only a trace amount of damaged peptide (during phase transition) is needed to produce a trace amount of bad catalyst. This bad catalyst can trigger a large or small amount of multimerization later, depending on the storage conditions.

5. Conclusions

It is critical to optimize the lyophilization cycle when the ratio of mannitol to peptide changes. Otherwise, it can trigger multimer formation.

Even while ANP is a disulfide-linked peptide containing no free cysteine, two kinds of multimers can form, one reducible and the other non-reducible. The reducible multimers were the disulfide-linked multimers. The non-reducible dimer was covalently linked by the side chain of tyrosine in one molecule and the dehydroalanine intermediate in another molecule.

The resolving power of the SEC method enabled us to purify the different multimers. Therefore, these multimers can be further analyzed by different characterization techniques to understand the mechanism of their formation. By using this SEC method quantitatively, we can optimize the lyophilization cycles, select the buffer and bulk agents, and predict the shelf life.

The formation of these multimers were diminished by the addition of an annealing step with an optimal ratio of mannitol to peptide in the lyophilization, and/or by the increase of the acetate buffer concentration in the formulation.

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