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International Journal of Pharmaceutics 351 (2008) 1–7
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Stabilization of a polypeptide in non-aqueous solvents

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Received 5 May 2006; received in revised form 7 September 2007; accepted 10 September 2007
Available online 15 September 2007

Abstract

A pituitary adenylate cyclase-activating peptide (PACAP) analogue (HSDAVFTDNYTRLRKQVAAKKYLQSIKNKRY, P66) was formulated in several non-aqueous solvents in anticipation of improved shelf-life stability. However, the stability of this peptide in these solvents was found to be as poor as in an aqueous solution. The major degradation reaction in non-aqueous solvents was dimer formation. The proposed mechanism for dimerization was a nucleophilic attack of a basic amino acid on cyclic imide formed by dehydration or deamidation of Asp or Asn. Two approaches were found to be effective in stabilizing the peptide in non-aqueous solvents: (1) acidification of the peptide and (2) use of zinc chloride in the formulation. Stabilization could be attributed to reduction of the nucleophilicity of the reactive groups through protonation and metal–peptide interaction through chelation. The stabilization approaches are applicable only in a non-aqueous environment for this peptide, and possibly for other peptides with similar reactive moieties.

Keywords: Peptide stability; Non-aqueous solvent; Deamidation; Zinc stabilization; Dimerization; PACAP

1. Introduction

Stability studies of biologically active peptides are usually conducted in an aqueous solution (Pontiroli, 1998). Although non-aqueous solvents have been shown to modulate peptide synthesis (Gololobov et al., 1994; Haensler and Arnold, 2000) or to facilitate peptide digestion (Russell et al., 2001), the stability of peptides in non-aqueous solvents is an under-explored field partly due to limited practical applications of these systems (Stevenson, 2000). Although non-aqueous solvents usually exhibit a detrimental effect on the conformation stability of proteins, these solvents may or may not affect the stability of peptides as there is limited or no tertiary structures in peptides. In fact, these solvents often improve the stability of peptides by promoting formation of secondary structures (Zou and Sugimoto, 2000; Kozin et al., 2001). Peptide deamidation can be modestly inhibited in an aqueous solution upon addition of an organic solvent such as glycerol (Li et al., 2000), and ethanol or dioxane (Brennan and Clarke, 1993). A 9-amino acid LHRH analogue, leuprolide, has been formulated in dimethyl sulfoxide (DMSO) as a controlled release product, Viadur®, partly because its overall storage stability in DMSO is better than in water (Stevenson et al., 1999; Hall et al., 1999). Therefore, when the stability of a peptide in water is a concern, the use of a non-aqueous solvent may be an alternative.

The pituitary adenylate cyclase-activating peptide (PACAP) is a class of hormones with less than 40 amino acids. By binding to three different types of receptors R1, R2, and R3, PACAP initiates a variety of pharmacological activities, including glucose-dependent release of insulin (R3). To design a potential drug candidate with selective R3 activity, a PACAP mutein was synthesized (designated as P66), which showed ∼150-fold selectivity for R3 over R2 receptors with no significant activation of R1 receptors (Tsutsumi et al., 2002). In our stability studies, we found that P66 was not stable in an aqueous solution, as initially suspected. Its stability in non-aqueous solvents was then evaluated. Unexpectedly, the stability of this peptide in a few non-aqueous solvents was as poor as in an aqueous solution.

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doi:10.1016/j.ijpharm.2007.09.012

To improve the stability of P66 in these non-aqueous solvents and to increase its chance of being developed as a potential drug candidate, we examined the instability of P66 both in aqueous and non-aqueous solutions and a variety of stabilization strategies. Two of these strategies—metal chelation and acidification of the peptide, turned out unexpectedly to be very effective. As such, a patent application was filed (Wang et al., 2005). We believe that stabilization of peptide in non-aqueous solvents have not been reported in the literature. The observed stabilization also contributed to the understanding of degradation mechanism.

2. Materials and methods

2.1. Materials

P66, a 31 amino acid peptide (HSDAVFTDNYTRL-RKQVAAKKYLQSIKNKRY), was chemically synthesized (MW = 3742) and purified to over 98% by UCB Bioproducts (Braine-I'Alleud, Belgium). This lyophilized product contains about 8% moisture and 7% acetic acid. Based on this, the molar ratio of P66:acetate is approximately 1:5. Organic solvents and chemicals were used as received, including dimethyl sulfoxide (DMSO, 99.9%), monothioglycerol, $ZnCl_2$, $MgCl_2$, $CaCl_2$, hydrochloric acid and phosphoric acid (Spectrum Chemicals, Gardena, CA), propylene glycol (J.T. Baker, Phillipsburg, NJ), trifluoroacetic acid (TFA) (Pierce, Rockford, IL), diethanolamine (Sigma, St. Louis) and (Spectrum Chemicals, Gardena, CA).

2.2. Preparation of stability samples

P66 stability samples were prepared by directly dissolving the peptide in a proper solvent at 2 mg/mL based on peptide content. The bulk peptide solution was aseptically divided into 2-mL screw-capped (with an o-ring) sterile polypropylene vials. These vials were incubated in an oven at 40 °C and samples were removed periodically for assay. All the stability samples were frozen at −70 °C if they were not immediately analyzed.

2.3. Peptide stabilization in non-aqueous solvents

Two stabilization strategies were examined. One was acidification of the peptide and the other was addition of formulation excipients. To acidify the peptide, 0.1% HCl, TFA, or H_3PO_4 solution was cooled to 2–8 °C and mixed with P66 at a P66:acid molar ratio of 1:10. After mixing, the cold P66 solutions were immediately placed inside a pre-cooled freeze-drier and were lyophilized. The lyophilized material was further incubated in a desiccator containing P_2O_5 for at least 1 day. The acidified and then dried peptide was dissolved in a non-aqueous solvent for stability studies as described before.

Several metal salts, including $ZnCl_2$, $MgCl_2$, and $CaCl_2$, were used as potential formulation excipients and mixed with the peptide for stability studies. Each one of these salts was first dissolved at 1 mM in a non-aqueous solvent. P66 was then dissolved in these solutions at 2 mg/mL. Stability studies were conducted as previously described.

2.4. Reverse phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC analysis was conducted at room temperature on a HP 1100 or 1050 HPLC (Hewlett Packard, Pleasanton, CA) with a C-18 column (150 mm × 4.6 mm, 5 μm, 300 Å, Vydac, Hesperia, CA). The samples were monitored at 280 nm. The mobile phase contained solvent A (10% acetonitrile, 90% water, and 0.1% TFA) and solvent B (90% acetonitrile, 10% water, and 0.1% TFA). Gradient elution was used, starting from 12.5% B and ending at 90% B. This method is suitable to measure the amount of P66, however, does not offer complete recovery of all degradants, and the degradants were not well separated.

2.5. Nuclear magnetic resonance (NMR)

NMR experiments were performed on a Bruker DMX-600 spectrophotometer equipped with a triple gradient inverse triple axis probe. Approximately 600 μL of peptide sample at 2 mg/mL, in deuterated DMSO or deuterium oxide, with or without $ZnCl_2$, was measured in 5 mm i.d. WILMAD NMR tubes at 300 K and with 128 scans using a relaxation delay of 3 s. A two-dimensional gradient enhanced ^1H–^1H homonuclear correlation (COSY) experiment was applied to identify the simple aromatic coupling networks of the peptide.

3. Results

3.1. Stability of P66 in non-aqueous solvents

To formulate peptide in non-aqueous solvents, a peptide has to be soluble in these solvents at a desirable concentration. Therefore, the solubility of P66 was screened initially in more than 20 non-aqueous solvents or such solvent mixtures. Several solvents were found to be able to dissolve P66 easily, including DMSO, propylene glycol, and monothioglycerol. We compared the stability of P66 at 2 mg/mL in these solvents at 40 °C. Unexpectedly, P66 degraded rapidly in all these solvents (Fig. 1). More than 90% of P66 degraded in propylene glycol after 7 days at 40 °C. DMSO is the most stable solvent for P66 but still, 90% degraded after 13 weeks.

Based on mass spectroscopy analysis of the collected sample fractions after RP-HPLC separation, the major degradation product was a dimer. Other degradation products include dehydrated, des-HS and des-HSD peptides and isomers. The seperation and identification of these degradation products, and its associated degradation mechanism—i.e. dehydration or deamidation of Asp or Asn followed by a nucleophilic attack of a basic amino acid, have been recently published (Severs and Froland, in press).

3.2. Stabilization of P66 by acidification

There are two Asp and two Asn residues in P66 that were identified as potential dehydration and deamidation sites (Severs and Froland, in press). Formation of the degradation intermediate, cyclic imide (aka succinimide or Asu), at these sites may

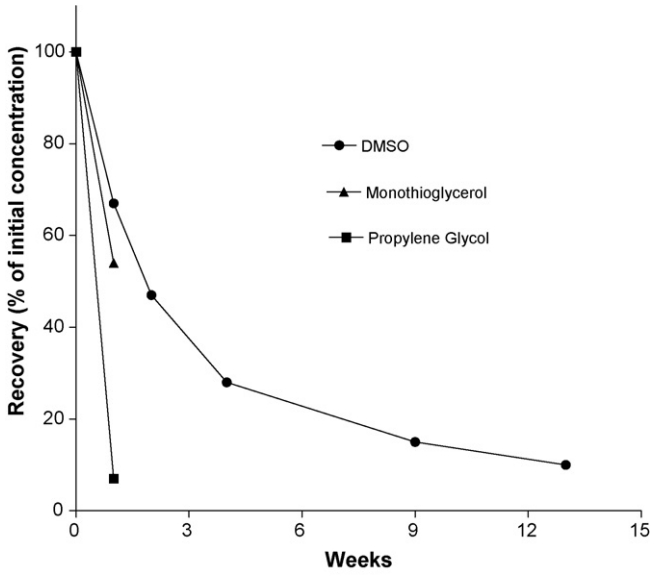

Fig. 1. Degradation of P66 in pure non-aqueous solvents. Stability samples were incubated at 40 °C and analyzed by RP-HPLC.

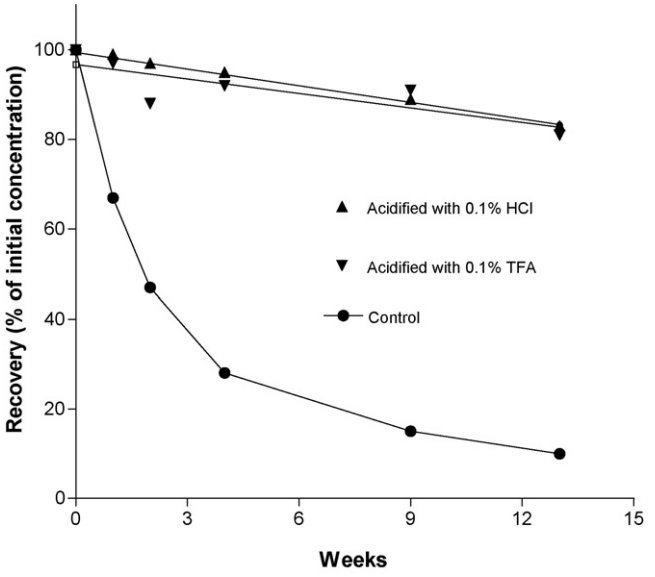

Fig. 2. Effect of acidification on the stability of P66 at 2 mg/mL in DMSO. Stability samples were incubated at 40 °C and analyzed by RP-HPLC.

result from a nucleophilic attack of the amide nitrogen on the side chain carbonyl carbon. Acidification of the peptide would theoretically reduce the nucleophilicity of the attacking group(s) and consequently, reduce the peptide degradation. To prove this hypothesis, we acidified the peptide with acid solutions. The pH of the peptide solution, prior to lyophilization, was dropped to 2.1 and 2.2, respectively, in the presence of 10 M excess of 0.1% HCl and TFA. To prevent potential acid-induced hydrolysis in an aqueous solution, the peptide was acidified with a cold acid solution and the solution was immediately dried by lyophilization (see Section 2). The lyophilized peptide was then dissolved directly in pure DMSO. Because the acidification and re-lyophilization processes were fast and conducted at a low temperature, no detectable hydrolysis of P66 was observed during the preparation processes. The stability of the acidified P66 in DMSO is shown in Fig. 2. Indeed, acidification of P66 with HCl or TFA resulted in a significant improvement in storage stability in neat DMSO.

3.3. Stabilization of P66 by formulation excipients

Another simple and effective way of stabilizing a peptide in an aqueous solution is to use formulation excipients as stabilizers. However, in non-aqueous solvents, the solubility of these excipients can be very limited and therefore, may not exert a desirable stabilizing effect in these solvents. Among these potential peptide/protein formulation excipients are metal salts. Metal salts are often used to stabilize certain peptides or proteins in an aqueous solution by chelation reaction or simple ionic interaction. Since P66 does contain a terminal histidine residue and two Asp residues that can potentially interact with a metal salt, we examined the peptide stability in the presence of $ZnCl_2$, $MgCl_2$, and $CaCl_2$ at 1 mM in neat DMSO at 40 °C. All of these metal salts at this concentration were able to be dispersed well with no visible particulates in DMSO. We found that P66 was significantly stabilized in the presence of 1 mM $ZnCl_2$ and less than 40% of P66 degraded in 13 weeks at 40 °C relative to approximately 90% for the control formulation (Fig. 3). Addition of $MgCl_2$ or $CaCl_2$ did not have any significant effect on the stability of the peptide.

The significant stabilization of P66 by $ZnCl_2$ in a non-aqueous solution prompted us to investigate whether the stabilization effect is also operative in an aqueous solution. The stability of P66 at the same concentration in pure water was then examined in the presence of 1 mM $ZnCl_2$. Unexpectedly, zinc did not show any stabilizing effect (Fig. 4). In fact, zinc seemed to accelerate the peptide degradation at a later stage during storage. The major degradation products of P66 in an aqueous solution include isomers, dehydrated products, des-H, des-HS, des-HSD

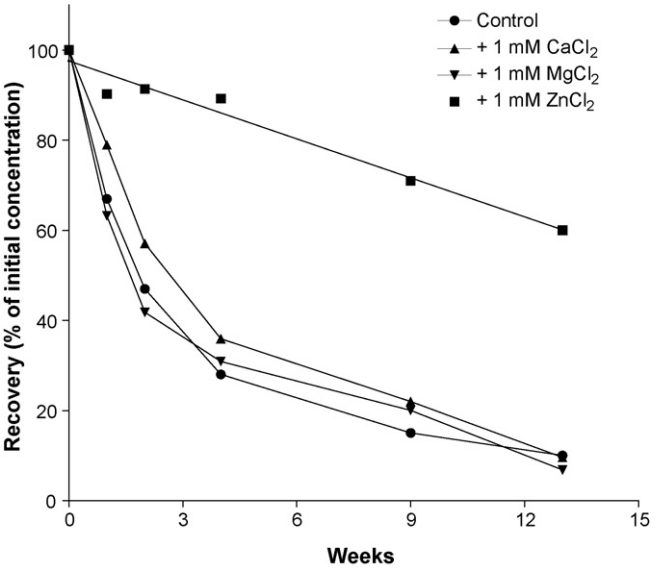

Fig. 3. Effect of metal salts on the stability of P66 at 2 mg/mL in pure DMSO. Stability samples were incubated at 40 °C and analyzed by RP-HPLC.

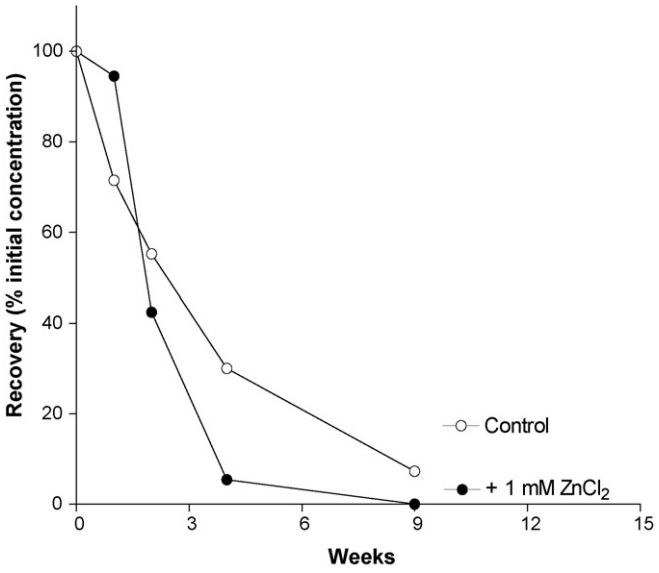

Fig. 4. Effect of 1 mM $ZnCl_2$ on the stability of P66 (2 mg/mL) in water during storage at 40 °C. Stability samples were diluted to 1 mg/mL before RP-HPLC analysis.

peptides, and no dimers were detectable (Severs and Froland, in press).

3.4. Investigations on the mechanisms of P66 stabilization by zinc chloride

The stabilization of P66 by zinc chloride suggests a possible chelation reaction between the transition metal and the peptide. To determine whether there is such an interaction, NMR experiments were performed on the peptide in deuterated DMSO with and without zinc chloride. Fig. 5 shows the aromatic region of the spectra, which exhibited significant line broadening as expected for a peptide of this size. In the presence of zinc chloride, the

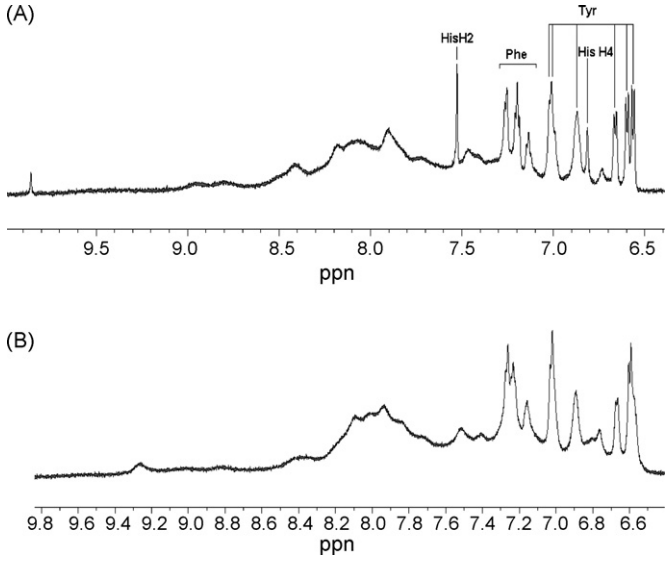

Fig. 5. NMR spectra of P66 at 2 mg/mL in deuterated DMSO in the absence (panel A) and presence (panel B) of 1 mM zinc chloride.

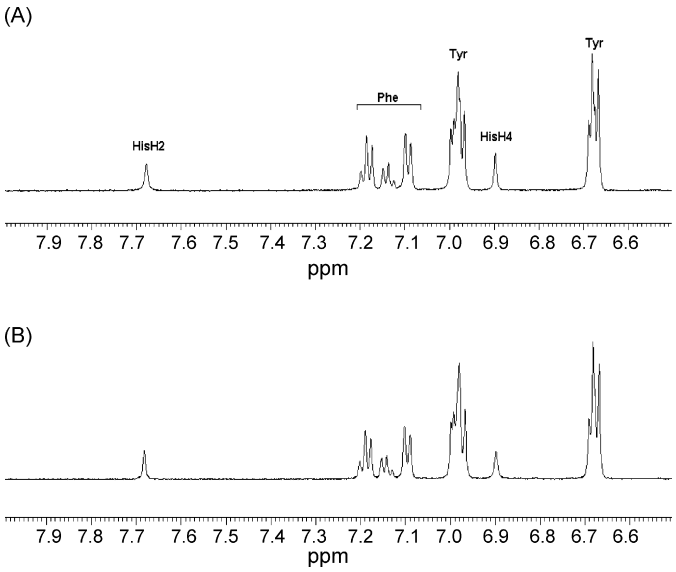

Fig. 6. NMR spectra of P66 at 2 mg/mL in deuterated water in the absence (panel A) and presence (panel B) of 1 mM zinc chloride.

imidazole H2 and H4 signals disappeared, suggesting that zinc chloride clearly interacts with the N-terminal histidine group through imidazole. In comparison, the aromatic NMR spectra of P66 in deuterated water with or without 1 mM zinc chloride are the same (Fig. 6), indicating no interaction or that the interaction may be simply too weak to be detected in an aqueous solution. On the other hand, the aliphatic region of the NMR spectrum in deuterated water in the presence of zinc chloride was different with changes in the chemical shift of Hα at residues Ser_2, Asp_3, Asn_9, and Tyr_{10} (data not shown). This result suggests that zinc chloride can still interact with these residues to some degree in an aqueous solution.

4. Discussion

4.1. Instability of P66

This study demonstrates that P66 is very unstable not just in water (Fig. 4) but also in several non-aqueous solvents (Fig. 1). The relative stability in these non-aqueous solvents are DMSO > monothioglycerol > propylene glycol. The degree of instability of P66 seems to be greater than what is expected of a peptide in general. Although interpretations for such "abnormal" peptide instability have been offered in the literature, none seem to be convincing. For example, the instability of vasoactive intestinal peptide (VIP), a closely related peptide structurally, was attributed to peptide autolysis (Mody et al., 1994). Since the N-terminal structure of this peptide contains the catalytic triad of serine proteases (His/Ser/Asp), one possibility for the poor stability is that P66 has certain degree of protease activity to catalyze its own degradation. To examine this possibility, we conducted the stability study of the peptide in DMSO in the presence of a proteinase inhibitor, phenylmethylsulfonyl fluoride. The stability of the peptide was not improved in its presence (data not shown). Therefore, it is unlikely that the instability is due to self-catalyzed degradation.

The major degradation pathway (dimer formation) for P66 in DMSO has been determined and the results have been published recently (Severs and Froland, in press). In this publication, LC/MS analysis of chymotrypsin-digested dimers identified a number of cross-linked peptide fragments with a loss of either H_2O or NH_3, suggesting that Asp and Asn were involved. In addition, these fragments were composed of segments having Asp or Asn with Arg or Lys residues. Thus, dimerization was proposed to initiate through dehydration at Asp or deamidation at Asn with concurrent formation of a cyclic imide, and followed by a nucleophilic addition of a basic amino acid such as Arg or Lys from another molecule. In comparison, the traditional ring opening reaction of the cyclic imide through addition of a hydroxide ion in aqueous solution results in formation of Asp and IsoAsp peptides. In the absence of hydroxide ions in non-aqueous environment, the amino groups from Arg or Lys residues would be the main nucleophiles and could initiate nucleophilic attack on the imide ring, forming predominant dimers.

4.2. Stabilization of P66 by acidification

It is well known that the formation of a cyclic imide begins with an intra-molecular, nucleophilic attack of an amide backbone nitrogen on the carbonyl carbon on the side chain of a reactive amino acid, such as Asn or Asp. Increasing the alkalinity of an aqueous solution will accelerate the nucleophilic attack thus facilitate the formation of a cyclic imide, as a basic condition favors deprotonation of the backbone nitrogen and the deprotonated nitrogen has a higher nucleophilicity (Oliyai and Borchardt, 1993; Capasso et al., 1993). Therefore, acidification of the peptide would reduce the reactivity of the backbone nitrogen and slow down the reaction. Indeed, deamidation at Asn is strongly pH dependent and increasing the solution pH caused a significant increase in deamidation of a model peptide (Capasso et al., 1993; Patel and Borchardt, 1990a,b).

We suspected that acidification of a non-aqueous solution would have a similar effect on the formation of cyclic imide as in an aqueous solution. This study clearly demonstrates that P66 can be stabilized significantly by acidification of the peptide in a non-aqueous solution. As demonstrated in an aqueous environment, protonation could decrease nucelophilicity of the backbone nitrogen and inhibit cyclic imide formation. More importantly, since the major degradation pathway is dimerization mediated by a key and non-reversible nucleophilic attack of a basic amino acid on the cyclic imide, protonation of the amino groups on the Arg or Lys residue under acidic conditions would effectively inhibit their nucleophilicity, consequently preventing dimer formation. Of course, we could not rule out the possibility of the secondary structure change under acidic conditions in non-aqueous solvents, leading to reduced degradation rate.

4.3. Stabilization of P66 by zinc

This study also demonstrates that P66 can be stabilized significantly by zinc chloride in DMSO. Since the other two non-transition metals failed to stabilize P66 in DMSO, zinc-induced stabilization of P66 may not be due to simple ionic interactions with the peptide. Instead, a chelation interaction between the peptide and zinc is likely responsible for the reduced chemical degradation of the peptide in a non-aqueous solvent. The altered NMR spectrum in the presence of zinc chloride in DMSO indicates such an interaction, which involves at least the N-terminal histidine residue in P66 in DMSO. It is a fact that such metal–peptide chelation interactions usually lead to NMR signal broadening, relocating, or disappearing in the extreme cases (Carter and Gardella, 2001; Jobling et al., 2001; Razmiafshari et al., 2001). In the presence of zinc chloride, the disappearance of the two aromatic H_2 and H_4 peaks derived from the imidazole ring suggests that the imidazole nitrogen is likely a chelation site of zinc. Other chelation site(s) may also exist as the overall NMR spectrum was changed in the aliphatic region.

The use of a metal chelation mechanism is one of the key strategies in designing stable proteins/peptides (Kellis et al., 1991; Klemba et al., 1995; Regan, 1993). Zinc is one of the most commonly used stabilizing metals and can easily form a complex with histidine residues in proteins/peptides for stabilization. For examples, zinc alone stabilizes a somatostatin analogue (Deyoung and Lien, 1984), insulin (Brange et al., 1997; Brange and Langkjaer, 1992), and α_1-antitrypsin (Vemuri and Yu, 1993). The metal-induced stabilization has been attributed to chelation-induced rigidification of the peptide (Haran et al., 1982) and/or altered formation of secondary structures (Omichinski et al., 1991; Yang et al., 2000; Kozin et al., 2001). Although our data did not show specifically how P66 molecules interact with zinc in DMSO other than it is in coordination with the side chain imidazole of histidine, several binding possibilities can be speculated based on the studies carried out in water. One possibility is that zinc specifically coordinates with the His 1 and Asp 3 residues of P66 in DMSO. Such an interaction was reported for a structurally similar compound, secretin (HSDFTFT. . .) in water, forming a secretin hexameric complex (Carpenter and Schiller, 1998). If a similar interaction exists for P66 in DMSO, the formation of degradation intermediate, cyclic imide, at Asp 3 could be inhibited. Another possibility is the formation of His–Zn–His bridge, linking two P66 molecules and altering the flexibility of the peptide. Such a bridge formation has been reported for amyloid peptide $A\beta_{1–40}$, which contains as many as three His residues in an aqueous solution (Miura et al., 2000). Yet another possibility is the formation of His–Zn–Ser bridge. Such a heterogeneous bridge has been proposed for the interaction between Zn and prion peptide, $PrP_{106–126}$, which contains one histidine residue (His_{111}) (Jobling et al., 2001). The last possibility is that zinc links His and another amino acid(s) in the distance to form a ring-like structure, as proposed for the interaction between Zn and prion peptide, $PrP_{106–126}$ (Jobling et al., 2001).

In an aqueous solution, however, we did not detect any interaction between zinc and the histidine imidazole in P66 by NMR. Although interaction seemed to exist between zinc and other aliphatic amino acids, the interaction was also probably weak, as no stabilization was observed. It has been reported that the affinity of Zn(II) for peptides containing no-more-than two His residues is generally weak (Carter and Gardella, 2001). Therefore, it is likely the zinc–P66 interaction is too weak to be detected by NMR. We speculate that a weak inter-

action in an aqueous solution is likely due to both a strong metal–water interaction and a strong water–peptide interaction. Reducing the relative amount of water (by increasing the amount of organic solvents) might weaken both interactions and enhance metal–peptide interactions, strong enough to be detectable (Sigel et al., 1985). On the other hand, such an interaction was detectable in an aqueous solution between Cu(II), a different transition metal, and a smaller PACAP-like peptide (HSDGI-NH_2) (Kowalik-Jankowska et al., 1999). It is possible that the binding affinity of copper is stronger than zinc for such peptides. This is the case for prion peptide, $PrP_{106–126}$, where its single histidine residue does not bind to Zn(II) as strongly as Cu(II) (Jobling et al., 2001).

In summary, we found that P66 is unstable during storage not only in an aqueous solution but also in several non-aqueous solvents. However, the final degradation products were different. While the major degradation products of P66 in an aqueous solution are N-terminal hydrolyzed peptides, dehydrated peptides and isomers, dimers are the major products of degradation in a non-aqueous solution (Severs and Froland, in press). The instability of this peptide in a non-aqueous solution seems to be an inherent property, rather than a result of autocatalysis. Two approaches are effective in stabilizing the peptide in DMSO. One is to acidify the peptide and the other is to use a formulation excipient, zinc salt in this case. Their stabilization mechanisms are proposed to be, respectively, reduction of the nucleophilicity of the reacting moieties through protonation and metal–peptide interaction through chelation.

Acknowledgements

People, who contributed to the data in this report, include John Guo for preparing the key stability samples, Dennis Chen and Joanne Severs for analyzing stability samples and identifying degradation products, Machiko Yagami for screening peptide solubility in non-aqueous solvents and preparing stability samples, and Michael Dumas and Wayne Froland for their valuable discussions.

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