Comparison of the Rates of Deamidation, Diketopiperazine Formation, and Oxidation in Recombinant Human Vascular Endothelial Growth Factor and Model Peptides

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ABSTRACT In this work, we examine the way in which stability information obtained from studies on small model peptides correlates with similar information acquired from a protein. The rates of deamidation, oxidation, and diketopiperazine reactions in model peptide systems were compared to those of recombinant human vascular endothelial growth factor (rhVEGF). The N-terminal residues of rhVEGF, a potent mitogen in angiogenesis, are susceptible to the aforementioned reactions. The degradation of the peptides L-Ala-L-Pro-L-Met (APM) and Gly-L-Gln-L-Asn-L-His-L-His (GQNHH), residues 1-3 and 8-12 of rhVEGF, respectively, and rhVEGF were examined at pH 5 and 8 at 37°C. Capillary electrophoresis and high-performance liquid chromatography (HPLC) stability-indicating assays were developed to monitor the degradation of the penta- and tripeptides, respectively. The degradation of rhVEGF was determined by tryptic mapping and quantified by RP-HPLC. The rates of degradation of both peptides and the protein followed apparent first-order kinetics and increased with increasing pH. The tripeptide APM underwent diketopiperazine formation (Ala-Pro-diketopiperazine) and oxidation of the Met residue, whereas the pentapeptide GQNHH degraded via the deamidation pathway. The results indicate that the rates of deamidation and oxidation of the protein are comparable to those observed in the model peptides at both pH values. However, the rate of the diketopiperazine reaction was slower in the protein than in the model peptide, which may be the result of differences in the *cis-trans* equilibrium of the X-Pro peptide bonds in the 2 molecules.

INTRODUCTION

Vascular endothelial growth factor (VEGF) has been identified as a heparin-binding polypeptide mitogen that plays an important role in physiologic and pathologic angiogenesis. It is secreted by ischemic tissues and has a highly specific role in the maintenance and induction of growth of the vascular endothelial cells (1-4). Analysis of the human cDNA clone of VEGF revealed that the protein exists in 4 different isoforms having 121 (VEGF₁₂₁), 165 (VEGF₁₆₅), 189 (VEGF₁₈₉), and 206 (VEGF₂₀₆) amino acid residues (5). The heterogeneity of VEGF was shown to arise from alternative exon splicing of a single VEGF gene (5). VEGF₁₂₁ is a weakly acidic polypeptide that fails to bind to heparin (6), while VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆ are more basic and bind heparin with increasingly greater affinities (7). Of these 4 species, VEGF₁₆₅ is the predominant molecular form produced in normal cells and tissues (8). The other isoforms, VEGF₁₂₁ and VEGF₁₈₉, are detected in the majority of cells and tissues that expresses the VEGF gene, while VEGF₂₀₆ is a rare form that has been identified in human fetal liver only (5,7).

The native VEGF is a basic, heparin-binding, highly conserved homodimeric protein, with a molecular mass of 46 kDa (1,9). The structure of the protein shows that it belongs to the cystine knot growth factor superfamily and is composed of an antiparallel homodimer, covalently linked by 2 disulfide bridges between Cys-51 and Cys-60 (10). Recombinant human vascular endothelial growth factor (rhVEGF) behaves in a manner similar to native VEGF in terms of its binding to heparin and its biological activity (5). rhVEGF is a homodimeric protein consisting of 165 amino acids per monomer with a molecular weight of 38.3 kDa and a pI of 8.5. The protein consists of 2 domains, a receptor-binding domain

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(residues 1-110) and a heparin-binding domain (residues 111-165) (11,12).

It was observed that around neutral pH values, the N-terminal sequence of rhVEGF is susceptible to deamidation, diketopiperazine formation, and oxidation (J.L. Cleland, R. Keck, and A.J.S. Jones, unpublished data). The major degradation pathway is the deamidation of the Asn-10 residue. Diketopiperazine products and oxidation products (ie, Met-3 residue) were observed upon storage of the protein.

In this study, we were interested in examining the way in which stability information obtained from studies on small model peptides correlates with that of the in vitro chemical stability of a protein. In order to achieve this objective, the rates of deamidation, diketopiperazine formation and oxidation of rhVEGF and model peptides were compared. The model peptides we chose consisted of sequences corresponding to sites in the protein where these reactions occurred. The tripeptide L-Ala-L-Pro-L-Met (APM) and pentapeptide Gly-L-Gln-L-Asn-L-His-L-His (GQNHH), which constitute residues 1-3 and 8-12 of rhVEGF, respectively, were the model peptides used in this study.

MATERIALS AND METHODS

Materials

The peptides APM and GQNHH were synthesized by California Peptide Research, Inc. (Napa, CA). Recombinant human vascular endothelial growth factor was produced by Genentech, Inc. (South San Francisco, CA). The protein bulk solution contains 2.4 mg/mL of rhVEGF, 104 mg/mL trehalose, 0.01% Tween-20, and 5 mM sodium succinate (pH 5.0). The protein bulk solution was concentrated using an Amicon Centriprep-30 concentrator (Beverly, MA) and lyophilized. Bovine lung aprotinin was purchased from Sigma Chemical Co. (St. Louis, MO), plasmin from Calbiochem-Novabiochem Corp. (La Jolla, CA), and TPCK-trypsin from Worthington Biochemical Corp. (Lakewood, NJ). High-purity urea was obtained from Pharmacia Biotech (Uppsala, Sweden) and low-peroxide Tween-20 from Karlshamms, USA (Columbus, OH). All other chemicals were of analytical grade and used as received. The water used in all studies was from a Millipore MILLI-QTM water system.

Separation of Receptor and Heparin Domains of rhVEGF

The proteolytic enzyme plasmin (EC 3.4.21.7), which has a broad spectrum of activity, was used to separate the 2 domains of the protein. The technique used was similar to that employed by Keyt et al (12). Plasmin was added to the protein (0.5% w/w) in 50 mM phosphate buffer (PBS) at pH 7.4 and incubated at 25°C for 24 hours. The digest was stopped by adding to the mixture a 10-fold excess of aprotinin, a potent inhibitor, with respect to plasmin. A POROS^R HE-2 heparin column (1.5 x 10 cm) operated at room temperature was used to separate the fragments. The mobile phase consisted of 10 mM PBS, pH 7.0 (solvent A) and 3.0 M NaCl in 10 mM PBS, pH 7.0 (solvent B). Elution was accomplished using a flow rate of 2.0 mL/min and a linear gradient of 5% to 20% solvent B in 5 minutes (Figure 1). The fractions containing the receptor binding domain were collected and dialyzed against phosphate buffer (pH 7.0).

Figure 1. HPLC chromatograms showing the separation of the receptor and heparin-binding domains of rhVEGF. The receptor domain of the protein elutes with the void volume, and the heparin domain elutes at 3 minutes.

Tryptic Digest

The receptor domain samples were reduced and carboxymethylated prior to treatment with trypsin (EC 3.4.21.4). Denaturation of the protein was carried out in 8 M urea in 360 mM Tris buffer, pH 8.6 containing 2 mM ethylenediaminetetraacetic acid (EDTA). The disulfide bonds were reduced with 1 M dithiothreitol and alkylated with 1 M iodoacetic acid.

After buffer exchange into 100 mM Tris buffer (pH 8.3) containing 2 mM CaCl₂, trypsin (2% w/w) was added to the solution. The samples were then incubated at 37°C for 4 hours. At the appropriate time, the digestion was stopped by adding 85% phosphoric acid to the mixture.

HPLC Analysis

Analyses of the tryptic fragments of rhVEGF were performed using reversed-phase high-performance liquid chromatography (RP-HPLC). The system used consisted of a Hewlett-Packard 1090 liquid chromatograph system with a diode array detection and a Turbochrom 3 Chromatography Workstation data acquisition system (Perkin Elmer, Cupertino, CA). Separation was performed using a Phenomenex Jupiter 300 SC-18 column (5 µm, 4.6 x 250 cm) operating at 40°C. The solvent systems consisted of 50 mM sodium phosphate, pH 3.5 (solvent A) and acetonitrile (solvent B). The tryptic fragments were separated using a linear gradient of 0 to 50% solvent B in 50 min. The flow rate was maintained at 1.0% mL/min and the detection wavelength was 214 nm. The fragments were collected from the analytical HPLC and analyzed by matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS).

Kinetic Measurements

Experiments were carried out in phosphate buffer at pH 5.0 and 8.0 at 37°C. A constant ionic strength of 0.5 M was maintained for each buffer by adding a calculated amount of NaCl. The buffers were prepared at the experimental temperature and the pH value for a given solution remained unchanged throughout the investigation.

Recombinant human vascular endothelial growth factor was dissolved in the appropriate buffer solution to yield an initial concentration of approximately 1 mM. Aliquots (750 µL) of the resulting solution were transferred to vials, sealed and stored at 37°C. At various times, a vial was removed, cooled to room temperature, and subjected to analysis.

The stability of the peptides was carried out under similar conditions as those of rhVEGF, in 100 mM phosphate buffer (I = 0.5) containing 0.01% Tween-20 at pH 5 and 8. The degradation of APM was monitored by HPLC with a Vydac C-18 column (4.5 x 250 mm, 300 Å, while the degradation of GQNH

was followed by a capillary electrophoresis stability-indicating assay (13).

RESULTS AND DISCUSSION

Characterization of the Tryptic Fragments

There are 7 tryptic sites in the monomer of the receptor domain of rhVEGF, resulting in 8 peptide fragments (Table 1). The RP-HPLC separation of the fragments is shown in Figure 2. The fragments were identified by MALDI mass spectroscopy. The T7 and T8 fragments were not detected, being single amino acid and dipeptide species, respectively. Located within the T1 fragment are the residues susceptible to deamidation, diketopiperazine formation, and oxidation. As such, significant changes occur in the peak area of this fragment over time, with the appearance of new peaks.

Figure 2. The RP-HPLC separation of the tryptic fragments of rhVEGF. The peaks were identified by mass analysis.

Table 1. Theoretical Tryptic Peptide Fragments of the Receptor-Binding Domain of rhVEGF

Tryptic Fragment	Molecular Weight*	Sequence
T1	1660.8	APMAEGGGQNHHEVVK
T2	958.1	FMDVYQR
T3	4092.7	SYCHPIETLVDFQEYPDIEYIFKPSCVPLMR
T4	3065.4	CGGCCNDEGLECVPTESNITMQIMR
T5	2229.5	IKPHQQQHIGEMSFLQHNK
T6	851	CECRPK
T7	146.2	K
T8	289.3	DR

*The cysteine residues are assumed to be carboxymethylated.

Kinetics of Degradation of the T1 Tryptic Fragment

Deamidation of the Asn-10 residue, oxidation of Met-3, and the diketopiperazine formation reaction occur within the T1 fragment of the protein. Figure 3 shows a typical time course of the disappearance of the T1 fragment and the appearance of the degradation products. The apparent first-order rate constants for the appearance of the degradation products were obtained by a least-squares fitting procedure of the time course profiles at pH 5.0 and 8.0 to the following equations.

$$[A] = A_0 \exp(-Kt) \quad (\text{Equation 1})$$

$$[B] = \{(k_1 A_0) (\exp(-k_5 t) - \exp(-Kt)) / (K - k_5)\} \quad (\text{Equation 2})$$

$$[C] = \{(k_2 A_0) (\exp(-k_4 t) - \exp(-Kt)) / (K - k_4)\} \quad (\text{Equation 3})$$

$$[D] = \{(k_3 A_0) (\exp(-k_6 t) - \exp(-Kt)) / (K - k_6)\} \quad (\text{Equation 4})$$

These equations describe Scheme 1, where A, B, C, and D represent the T1, T1-deamidated, T1-oxidized, and des (Ala-Pro) T1 fragments, respectively. The constants k_1 , k_2 , and k_3 represent the rates of formation of the deamidated, oxidized, and truncated species, and k_4 , k_5 , and k_6 are the rates of disappearance of these species as they are generated (Scheme 1). The results of the least-squares procedure are summarized in Table 2. At both pH values, deamidation is the predominant degradation pathway and the most sensitive to changes in the pH of the solution. These data suggest that the protein undergoes deamidation at a relatively rapid rate. In a separate report, we have shown that the rate of deamidation of the Asn-10 residue is acid-base catalyzed by a His residue located on its C-terminal side (13). The protein also undergoes oxidation faster than diketopiperazine formation, ie, approximately 1.3 times faster at the lower pH and 2.7 times faster at the higher pH value.

Scheme 1

Table 2. Summary of the Rate Constants* of Degradation of rhVEGF in 100 mM Phosphate Buffer (I = 0.5) at 37°C

Parameter	Rate Constants $\pm$ SD ($10^4 \times h^{-1}$)	
	pH 5.0	pH 8.0
k_1	5.62 (± 0.67)	41.8 (± 1.3)
k_2	0.49 (± 0.28)	1.24 (± 0.42)
k_3	0.65 (± 0.38)	3.37 (± 1.93)
k_4	0.84 (± 0.32)	3.22 (± 0.99)
k_5	5.99 (± 0.58)	36.1 (± 3.4)
k_6	5.12 (± 0.69)	35.3 (± 6.2)

*Rate constants are for the species shown in Scheme 1 and Equations 1-4.

Figure 3. Kinetic profile for the degradation of the T1 tryptic fragment (open square) at pH 8.0, I = 0.5 and 37°C: open circle, deamidated product; open triangle, oxidization product; open diamond, des (AP) T1 fragment. The points represent experimental data and the lines were generated by a non-least squares curve-fitting to Equations 1-3 and the degradation pathways shown in Scheme 1

Table 3. Calculated Rate Constants* for the Degradation of APM at 37°C

Parameters	Values $\pm$ SD ($10^4 \times h^{-1}$)	
	pH 5.0	pH 8.0
K	2.125 (± 0.110)	14.85 (± 0.30)
k_{dkp}	1.475 (± 0.278)	7.633 (± 0.516)
k_{ox}	0.429 (± 0.121)	2.598 (± 0.372)
k_{dkp}^{ox}	0.027 (± 0.101)	0.252 (± 1.34)

*Rate constants are for the species shown in Scheme 2.

Scheme 2

Table 4. Comparison of the Rate Constants for the Degradation of the Model Peptides and rhVEGF in 100 mM Phosphate Buffer at 37°C

Rate of Degradation of Model Peptides ($10^4 \times \text{h}^{-1}$)			Rate of Degradation of rhVEGF ($10^4 \times \text{h}^{-1}$)		
GQNHH	APM				
pH	Deamidation	DKP Formation	Oxidation	Deamidation	DKP Formation
5.0	6.08 (± 0.09)	1.48 (± 0.24)	0.48 (± 0.15)	5.62 (± 0.67)	0.49 (± 0.28)
					0.65 (± 0.38)
8.0	48.6 (± 0.12)	7.63 (± 0.64)	2.59 (± 0.72)	41.8 (± 1.3)	1.24 (± 0.42)
					3.37 (± 1.93)

Degradation of AMP and GQNHH

The tripeptide APM can undergo diketopiperazine reaction and oxidation of the Met residue (Scheme 2). The disappearance of the initial peptide and the appearance of degradation products followed first-order kinetics. The apparent first-order rate constants were calculated from semi-logarithmic plots (Table 3). The diketopiperazine and oxidation reactions were sensitive to changes in the pH of the solution, occurring more rapidly at the higher pH value. The rate of diketopiperazine formation from the oxidized product, APM(O), was negligible at both pH 5.0 and 8.0. The rate constant for disappearance of APM at pH 8 is significantly greater than the sum of the rate constants for diketopiperazine formation and Ala-

Pro-Met(O) formation, suggesting that an additional pathway(s) may be involved in the degradation of this peptide at this pH. Previously, we have shown that the rate of diketopiperazine formation is dependent on the ability of the X-Pro peptide bond to adopt a *cis* conformation (14). In the *cis* conformation, the attacking N-terminal amino nitrogen atom is in proximity to the amide carbonyl between the second and third amino acids, facilitating ring closure (15). The *cis-trans* equilibrium of an X-Pro peptide bond has been shown to be dependent on the nature of the flanking amino acid and on the charge distribution around the bond (16-18).

The rates of degradation of GQNHH by the various pathways described above are summarized in Table 4. The rate of deamidation of the peptide followed pseudo-first-order kinetics and increased with increasing pH, which is consistent with literature reports (19). At pH 5.0, the pentapeptide degraded exclusively to the Asp- and isoAsp-containing products in a 1:3.5 ratio. However, at pH 8.0, detectable levels of the Asn-His peptide bond hydrolysis products were observed. These hydrolytic products constitute less than 5 percent of the total degradation products and were not incorporated into the analysis of the data.

Comparison of the Stability Information of the Model Peptides and rhVEGF

The rates of deamidation, diketopiperazine formation, and oxidation of rhVEGF and the model peptides are summarized in Table 4. These results indicate that the rates of deamidation and oxidation of the protein are comparable to those observed in the model peptides at both pH values. However, the diketopiperazine reaction occurs at a rate approximately 4 times slower in the protein than in the tripeptide.

The N-terminal residues of rhVEGF were shown by nuclear magnetic resonance spectroscopy to be disordered in solution (20). Thus, the labile Asn residue, which exists in a highly flexible region of the protein, is expected to have behavior similar to a model peptide having the same sequence. It was, therefore, not surprising that the rate of deamidation of the protein was comparable to the rate of deamidation of the model peptide used in this study. In addition, the mobile N-terminus of the protein is

exposed to the solvent, so it was not unexpected that the rate of oxidation of the Met-3 residue was similar to that observed in the model peptide.

In the model peptide APM, the peptide bond preceding the Pro residue is free to rotate around its axis and to adopt the most favorable conformation. However, although the N-terminal residues of the protein are disordered, the electron cloud of the overall protein structure can influence the X-Pro bond. The charge distribution around the Ala-Pro peptide bond in the protein could result in a shift in the equilibrium of the *cis-trans* ratio of that bond and, consequently, alter the rate of the diketopiperazine reaction. Thus, predicting the rate of diketopiperazine reaction in proteins from model peptide data would be problematic, because one of the major factors in determining the rate of this reaction is the *cis-trans* equilibrium of the X-Pro peptide bond.

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