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Capillary electrophoretic study of the degradation pathways and kinetics of the aspartyl model tetrapeptide Gly-Phe-Asp-GlyOH in alkaline solution

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ABSTRACT

The aim of the present study was the investigation of the isomerization and epimerization kinetics of the aspartyl tetrapeptide Gly-Phe-Asp-GlyOH at alkaline conditions. Incubations of the model tetrapeptide in sodium borate buffer, pH 10 and ionic strength 0.2 M, at 25 °C and 80 °C were analyzed by a validated CE–UV assay and fitted according to a pharmacokinetic model. CE–ESI-MS was used for peptide identification. Enantiomerization and isomerization of the aspartyl residue of the model tetrapeptide was observed under all experimental conditions applied. Differences in the velocity and the ratios of the rates of the degradation reactions indicated different effects of temperature on the individual reactions. At 80 °C, a rapid formation of β-Asp and D-Asp containing isomers from Gly-L-Phe-α-L-Asp-GlyOH was monitored. Rate constants of the hydrolysis of the succinimide (Asu) intermediate generally exceeded the formation of the intermediate from α/β-Asp peptides. A higher rate constant was observed for the enantiomerization from L-configured Asu compared to D-Asu. At 25 °C, epimerization and isomerization equilibrium was not reached within 5208 h. Compared to 80 °C different ratios of the individual reaction rates were noted. Moreover, inversion of the sequence of the first 2 amino acids was noted as a minor side reaction at 80 °C.

1. Introduction

Peptides and proteins are prone to chemical and physical degradation like deamidation, isomerization, and enantiomerization, as well as denaturation, and surface adsorption due to certain hot spots within the peptide sequence [1]. Two amino acids that are known to be among the most unstable amino acids are asparagine and aspartic acid [1–4]. Several groups demonstrated the formation of a five membered succinimide ring playing a central role in enantiomerization and isomerization of Asp and Asn residues [2–5]. This aminosuccinimide (Asu) is generated through an intramolecular nucleophilic attack of the side chain carbonyl group by the α-nitrogen of the peptide backbone amide liberating water or ammonia in case of Asp and Asn, respectively. Compared to the relative rate of Asu formation of an Asp and Asn residue, peptides containing aspartyl instead of asparagine react slower due to the fact that a nucleophilic attack of the backbone amide nitrogen is in principle only possible for a non-dissociated carboxyl group [4]. Asu formation being the rate limiting step in the enantiomerization and isomerization reactions depends on several factors, e.g., primary sequence, temperature, buffer type and pH [5–9]. Generally, Asu formation occurred faster in the presence of neighboring amino acids located at the C-terminus of the aspartyl residues with decreasing steric hindrance and lower conformational space. Afterwards, hydrolysis of the Asu intermediate yields either native Asp or iso-Asp peptides favoring the β-linkage by a ratio of 3–5:1 [2,3]. Furthermore, the cyclic imide can also epimerize leading to D-Asp containing peptides resulting in a 10^5 times faster enantiomerization rate due to the increased acidity of the α-carbon compared to free aspartic acid [3,10].

While Asp isomerization has been documented in many papers as summarized, for example, in [1,2,11–13], Asp enantiomerization has been less frequently addressed. β-Asp formation and concomitant enantiomerization were detected in synthetic peptide drugs such as klerval (N-ethyl-N-[1-oxo-4-(4-piperidinyl)butyl]glycyl-L-α-aspartyl-3-cyclohexyl-L-alaninamide) [14] and pramlintide [15] as well as natural proteins. Accumulation of β-Asp and D-Asp in human tissues including tooth dentin [16], βB2-crystallin [17], keratin in skin [18], and collagen in bone [19] has been described as an age dependent process and can be implicated in diseases [20]. Collins et al. determined D-Asp accumulation to date paleontological material [21]. Moreover, aspartyl isomers and enantiomers can

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be found in neurofibrillary tangles and plaques in Alzheimer's disease [22,23]. However, kinetic analysis of the enantiomerization processes is scarce.

Geiger and Clarke [3] reported significant Asp enantiomerization concomitant with isomerization studying the model hexapeptide Val-Tyr-Pro-Asn-Gly-AlaOH at pH 7.4. They suggested that enantiomerization of Asp or Asn proceeded mainly via the succinimide intermediate and determined identical rate constants for the corresponding epimerization reactions while L-Asu and D-Asu hydrolyzed at different rates. In contrast, studies of the model peptides Gly-Gln-L-Asn-L-Glu-GlyOH and Gly-Gln-D-Asn-L-Glu-GlyOH at pH 10 and 70 °C implied a competing epimerization at the stage of a tetrahedral intermediate [24]. Furthermore, Sadakane et al. detected isomerization and enantiomerization in recombinant human αA-crystallin protein at three specific aspartyl residues [25]. Although RP-HPLC separation of α/β-L/D-Asp peptides was achieved only isomerization was analyzed kinetically. Moreover, D-Asp and β-D-Asp peptides were observed during the investigation of the synthetic peptide klerval under neutral and basic conditions at 80 °C including the determination of the rate percent of the corresponding degradation reactions [14].

Studying the isomerization and epimerization of the model tripeptide Phe-Asp-GlyOH at elevated temperature Conrad et al. observed the formation of diketopiperazines as a competing reaction to isomerization and epimerization at pH 10 [26] as well as pH 2.0 and 7.4 [27]. Diketopiperazine formation has also been found as a significant degradation pathway of the related Asn peptide Phe-Asn-GlyOH by DeHart and Anderson [28]. This reaction appeared to be due to the attack of the N-terminal amino group on a carbonyl group of the Asu intermediate. It should not play a role when Asp (or Asn) is not in position 2 of the amino acid chain of a peptide. Therefore, the tetrapeptide Gly-Phe-Asp-GlyOH was synthesized and studied with regard to Asp isomerization and enantiomerization. using a capillary electrophoresis assay. Although isomerization is known to proceed relatively slow at high pH due to the dissociation of the side chain carboxyl group of Asp, pH 10 was selected in order to "isolate" the isomerization reaction from competing reactions such as backbone hydrolysis which would occur, for example at pH 7.4. As the reactions proceeded very slowly at 25 °C, 80 °C was studied primarily. The reactions were monitored using a capillary electrophoresis (CE) assay because CE has been proven to be a powerful analytical method for the separation and analysis of peptides including peptide stereoisomers [29–32].

2. Experimental

2.1. Chemicals

Fmoc-L-PheOH, Fmoc-L-Asp(OtBu)-OH, Fmoc-D-Asp(OtBu)-OH, Fmoc-L-AspOtBu, Fmoc-D-AspOtBu, Fmoc-GlyOH and Wang resin were obtained from Bachem AG (Heidelberg, Germany). Gly-L-Phe-α-L-Asp-GlyOH, Gly-L-Phe-α-D-Asp-GlyOH, Gly-L-Phe-β-L-Asp-GlyOH, and Gly-L-Phe-β-D-Asp-GlyOH were prepared by standard solid-phase peptide synthesis using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate as coupling reagent and 1-hydroxybenzotriazole as additive. Gly-L-Phe-L-Asu-GlyOH was synthesized by treatment of Gly-L-Phe-α-L-Asp-GlyOH with 1 mol/L HCl in acetic acid according to [33]. The crude peptides were purified by preparative HPLC using an acetonitrile/0.1% (v/v) aqueous trifluoroacetic acid (TFA) gradient. The respective fractions were combined and lyophilized. The peptides were characterized by mass spectrometry, HPLC and capillary electrophoresis (CE). The purity was at least 98% (HPLC and CE).

HPLC grade acetonitrile, acetic acid and TFA were purchased from Merck (Darmstadt, Germany), ethylamine and 1 mol/L HCl in acetic acid was from Sigma–Aldrich (Taufkirchen, Germany) while tris(hydroxymethyl)aminomethane (TRIS) and p-aminomethyl benzoic acid (PAMBA) were from Fischer Scientific (Schwerte, Germany). All other chemicals were of analytical grade and used as obtained. Purified water was prepared by a Milli-Q Direct 8 system (Millipore, Schwabach, Germany).

2.2. Capillary electrophoresis

CE analysis was carried out using a Beckman P/ACE MDQ (Beckman Coulter, Krefeld, Germany) equipped with a UV–vis spectrophotometric diode array detector and a 40 cm/50.2 cm, 50 μm i.d., 375 μm o.d. fused-silica capillary (BGB Analytik Vertrieb, Schloßböckelheim, Germany) thermostated at 20 °C. 350 mM acetic acid adjusted to pH 4.5 by addition of 2 M TRIS was the background electrolyte. All buffers were filtered (0.2 μm) and degassed by sonication. Between runs, the capillary was flushed for 1 min with 0.1 M sodium hydroxide and 3 min with the background electrolyte using a pressure of 690 kPa. UV detection was performed at 215 nm at the cathodic end of the capillary. The applied voltage was 25 kV. Samples were injected hydrodynamically at a pressure of 3.5 kPa for 5 s. PAMBA was used as internal standard to compensate for injection errors and monitor changes in the electroosmotic flow. Peak identification was achieved by co-injection of synthetic reference compounds and by CE-electrospray ionization mass spectrometry (CE–ESI-MS).

CE–ESI-MS measurements were performed on a 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) connected to a quadrupole-time-of-flight mass spectrometer (QqTOF MS, micrOTOFQ, Bruker Daltonik, Bremen, Germany) via a sheath liquid interface from Agilent Technologies (Waldbronn, Germany). The background electrolyte consisted of 450 mM acetic acid adjusted to pH 4.5 by ethylamine. A 62 cm long fused silica capillary with an inner diameter of 30 μm was used. A few millimeters of the polyimide coating of the capillary were burned off at the outlet tip and cleaned with 2-propanol. A voltage of 18 kV and a pressure of 3 kPa were applied. For sample injection a pressure of 7 kPa was operated for 12 s. ESI was performed in the positive mode, the MS inlet was set to 4500 V (sprayer on ground). The sheath liquid consisting of isopropanol-water (1:1, v/v) containing 1% acetic acid (v/v) was supplied by a syringe pump (Cole-Parmer, Illinois, USA) equipped with a 5 mL syringe (5MDF-LL-GT, SGE Analytical Science Pty Ltd, Melbourne, Australia) at a flow rate of 4 μL/min. Nebulizer gas pressure was set to 20 kPa. The drying gas (nitrogen) flow rate was set to 4 L/min, the drying temperature was 170 °C. As collision gas argon was chosen. Sodium acetate clusters were used for calibration. The mass range for MS spectra with a time resolution of 1 s was m/z 50–700. Both, low fragmentation spectra (fragmentation voltage 7 eV) and high fragmentation spectra (fragmentation voltage 15 eV) were acquired in subsequent analyses. Data processing was achieved with the DataAnalysis 3.4 software (Bruker Daltonik, Bremen, Germany). The fragments were labeled according to the standard nomenclature rules for peptides [34,35].

2.3. Kinetic studies

Incubations were performed in sodium borate buffer (pH 10; 50 mM). 50 mM boric acid was adjusted to pH 10 at 25 °C by addition of 1 M NaOH, the actual pH of the sodium borate buffer at 80 °C can be estimated using the coefficient for borate dpK_a/dT of $-0.008\ K^{-1}$ [36]. Each buffer was adjusted to an ionic strength of 0.2 M using solid sodium chloride. The concentration of the peptides was 6.0 μmol/mL. 0.15 μmol/mL PAMBA was added as internal standard in order to compensate for solvent evaporation. 70 μL aliquots were withdrawn at selected time intervals and diluted with 140 μL of ice-cold 0.1 M phosphoric acid. The