J|A|C|S
COMMUNICATIONS

Published on Web 08/28/2003

Racemization of an Asparagine Residue during Peptide Deamidation

Bei Li, Ronald T. Borchardt, Elizabeth M. Topp, David VanderVelde, and Richard L. Schowen*

Department of Pharmaceutical Chemistry, Simons Laboratories, The University of Kansas, 2095 Constant Avenue, Lawrence, Kansas 66047

Received May 12, 2003; E-mail: rschowen@ku.edu

The asparagine residue of the pentapeptide Gly-Gln-Asn-Glu-Gly (GQNEG) undergoes partial racemization at its C_α center during deamidation to Gly-Gln-Asp-Glu-Gly (GQDEG) and GQ-iso-aspartyl-EG (GQ-NHCH(CO_2^-)CH_2CH_2CO-EG) in aqueous sodium bicarbonate/carbonate buffer at pH 10, 70 °C. Figure 1 shows experiments in which GQ-L-Asn-EG transiently forms GQ-D-Asn-EG and in which GQ-D-Asn-EG transiently forms GQ-L-Asn-EG as all species undergo conversion to the deamidated products. Figure 2 shows the formation of partially epimerized products in these reactions. The products consist of a mixture of iso-aspartyl and aspartyl peptides with the iso-aspartyl species dominating in a 3:1 ratio, as is customarily observed.[1−3]

It has been known[1−4] that aspartate residues in peptides and proteins can cyclize to a succinimide species where the stereoelectronic relationship of the α-CH bond and the adjacent carbonyl group favors rapid, reversible fission of the CH bond by reaction with bases. This reversible dehydronation leads to a wholly or partially racemized succinimide residue, which then can open hydrolytically to generate similarly racemized aspartate and iso-aspartate residues in the product peptides. Asparagine residues can also cyclize to form a succinimide residue with expulsion of ammonia from the side-chain amide function. Racemization of the succinimide moiety then also leads to epimerized aspartyl and iso-aspartyl product peptides.

Radkiewicz, Zipse, Clarke, and Houk[4] have recently described the importance of these phenomena in medicine, in food chemistry, and in archaeological and palaeontological dating. They made an extensive study of the racemization mechanism by ab initio quantum mechanical methods and thoroughly characterized the structural and electronic properties of the succinimide intermediate that underlie its significance as the locus of racemization.

The transient appearance of a peptide containing a racemized asparagine residue, as in Figure 1, is unexpected because the expulsion of ammonia in formation of the succinimide species is irreversible in dilute aqueous solution, the ammonia product being so low in concentration that re-attack on the succinimide is prohibitively slow. There is thus no opportunity for return from an epimerized succinimide-peptide to a reactant Asn-peptide.[5]

We suggest that racemization can occur at the stage of the tetrahedral intermediate (TI) for formation of the succinimide residue, as shown in Scheme 1. This intermediate possesses a carbonyl group at least partly aligned for activation of the α-CH bond, and racemization of the tetrahedral intermediate would permit return from the epimerized state to the reactant Asn-containing peptide. In Scheme 1, rate constants for a given process are assumed to be approximately equal for all diastereomers.

Application of the steady-state approximation to both TI-peptide and succinimide (Asu)-peptide results in eqs 1 and 2 for the fractions

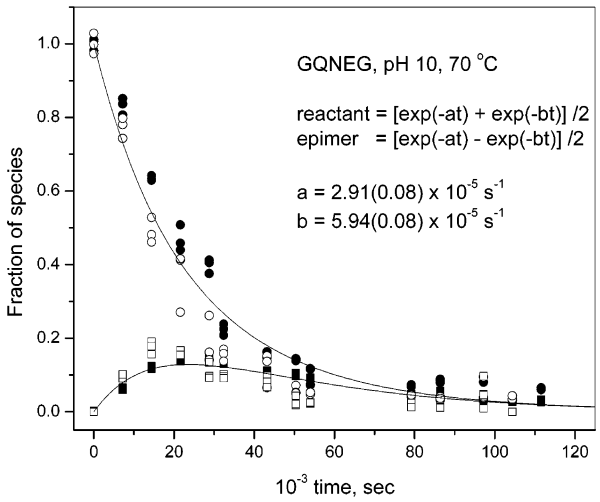


Figure 1. Transient formation of epimeric peptides during the deamidation of GQNEG. Filled circles depict the fraction of total pentapeptide concentration present as GQ-L-Asn-EG when this species was the initial reactant. Use of total pentapeptide species at any time rather than the initial reactant concentration corrects for up to 20% of hydrolytic fragmentation during deamidation. Open circles depict the fraction of total pentapeptide concentration present as GQ-D-Asn-EG when this species was the initial reactant. Filled squares show the transient appearance of GQ-D-Asn-EG when GQ-L-Asn-EG was the initial reactant. Open squares show the transient appearance of GQ-L-Asn-EG when GQ-D-Asn-EG was the initial reactant. The solid lines are plots of eqs 1 and 2 with the values of the rate constants *a* and *b* shown in the figure.

of initial peptide present as the reactant and as its epimer at Asn, respectively.

$$\text{reactant} = [\exp(-at) + \exp(-bt)]/2 \quad (1)$$

$$\text{epimer} = [\exp(-at) - \exp(-bt)]/2 \quad (2)$$

$$a = k_c k_e/(k_o + k_e) \quad (3)$$

$$b = k_c(k_e + 2k_r)/(k_o + k_e + 2k_r) \quad (4)$$

A global least-squares fit of the data produces the values of *a* and *b* shown in Figure 1 and the curves drawn there. The fractions present as products with the same configuration at Asn as the reactant (e.g., [L-aspartyl-peptide] + [L-isoaspartyl-peptide] for an experiment beginning with L-Asn-peptide) and as their epimer ([D-aspartyl-peptide] + [D-isoaspartyl-peptide] for an experiment beginning with L-Asn-peptide) are given by eqs 5 and 6, respectively.

$$\text{product} = \{[1 - \exp(-at)] + ([1 - \exp(-bt)]/f(\rho))\}/2 \quad (5)$$

$$\text{epimer} = \{[1 - \exp(-at)] - ([1 - \exp(-bt)]/f(\rho))\}/2 \quad (6)$$

10.1021/ja0360992 CCC: $25.00

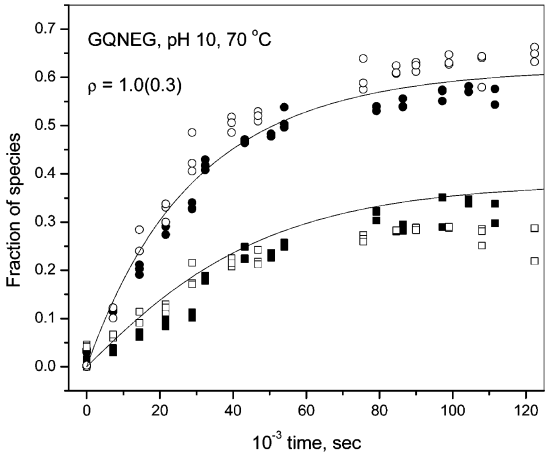


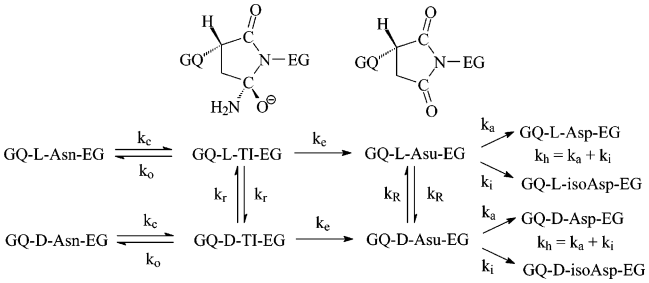
Figure 2. Formation of partially epimerized products in the deamidation of GQNEG. Circles represent the fraction of the total pentapeptide concentration (see caption to Figure 1) present as products with the same configuration at the aspartyl or iso-aspartyl residue as the initial reactant had at Asn (filled circles correspond to GQ-L-Asn-EG as initial reactant, open circles correspond to GQ-D-Asn-EG). Squares represent the fraction of the total pentapeptide concentration present as products with the configuration at the aspartyl or iso-aspartyl residue opposite to that the initial reactant had at Asn (filled squares correspond to GQ-L-Asn-EG as initial reactant, open squares correspond to GQ-D-Asn-EG). The lines are plots of eqs 5 and 6 with the values of *a* and *b* shown in Figure 1 and $f(\rho) = 4$.

Here, $f(\rho) = [1 + (2k_r/k_e)][1 + (2k_R/k_h)]$. The rate-constant ratios measure the tendency of the tetrahedral intermediate (k_r/k_e) or succinimide intermediate (k_R/k_h) to racemize relative to an irreversible elimination of ammonia or hydrolysis, respectively. Approximating $f(\rho)$ as $(1 + \rho)^2$ leads to $\rho = 1.0 \pm 0.3$ from a global fit of the product concentrations with *a* and *b* as shown in Figure 1. The resulting curves are shown in Figure 2.

The similarity of the data for GQ-L-Asn-EG epimerizing to GQ-D-Asn-EG and for independently synthesized GQ-D-Asn-EG epimerizing to GQ-L-Asn-EG tends to confirm that epimerization is indeed occurring at Asn. In a possible precedent to the present findings, Geiger and Clarke[6] found the rate of succinimide racemization quantitatively inadequate to account for total product epimerization with VYPNGA (37 °C, pH 7.4).

As a further test of Scheme 1, the NMR spectrum of GQ-L-Asn-NMe-EG, where NMe-E refers to an *N*-methylglutamyl residue, was monitored as the peptide was incubated in deuterium oxide under reaction conditions equivalent to those for which the data of Figures 1 and 2 were obtained. In this peptide, the nitrogen atom which must be dehydronated to form the tetrahedral intermediates shown in Scheme 1 has been methylated, precluding dehydronation.

Scheme 1



If epimerization of the Asn residue had nevertheless occurred in this peptide, deuterium would have been introduced at the C_α position and the corresponding ^1H signal would have decreased with time. In fact, no change in the NMR signal was detectable over 24 h, a result consistent with the hypothesis of Scheme 1 and with Asn as the only locus of peptide epimerization.

The tendency of Asn to racemize by this mechanism could be significant for the various fields mentioned above, but perhaps most important for the loss of protein function with age of the protein. Repair mechanisms have been identified[7] that reverse the damage of, for example, iso-aspartate formation in living organisms, but the more subtle potential route to loss of function identified here may be harder for the organism to address.

Acknowledgment. This work was supported by grant no. GM54195 from the National Institutes of Health.

Supporting Information Available: Complete experimental information and further discussion (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Aswad, D. W., Ed. *Deamidation and Isoaspartate Formation in Peptides and Proteins*; CRC Press: Boca Raton, FL, 1995.
(2) Schöneich, C.; Hageman, M. J.; Borchardt, R. T. In *Controlled Drug Delivery: Challenges and Strategies*; Park, K., Ed.; ACS Books: Washington, DC, 1997; pp 205−228.
(3) Robinson, N. E. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5283−5288.
(4) Radkiewicz, J. L.; Zipse, H.; Clarke, S.; Houk, K. N. *J. Am. Chem. Soc.* **1996**, *118*, 9148−9155.
(5) If nucleophilic attack by ammonia product were occurring, formation of iso-Asn-peptide would have been expected. None was observed. Further discussion of this point is given in the Supporting Information.
(6) Geiger, T.; Clarke, S. *J. Biol. Chem.* **1987**, *262*, 785−794.
(7) (a) Visick, J. E.; Cai, H.; Clarke, S. *J. Bacteriol.* **1998**, *180*, 2623−2629. (b) Smith, C. D.; Carson, M.; Friedman, A. M.; Skinner, M. M.; Delucas, L.; Chantalat, L.; Weise, L.; Shirasawa, T.; Chattopadhyay, D. *Protein Sci.* **2002**, *11*, 625−635.

JA0360992