ACS chemical biology

Articles

Cite This: ACS Chem. Biol. 2017, 12, 2875-2882

pubs.acs.org/acschemicalbiology

Sequence and Solution Effects on the Prevalence of D-Isomers Produced by Deamidation

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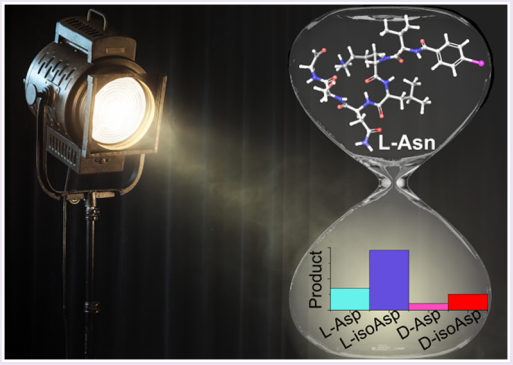
Supporting Information

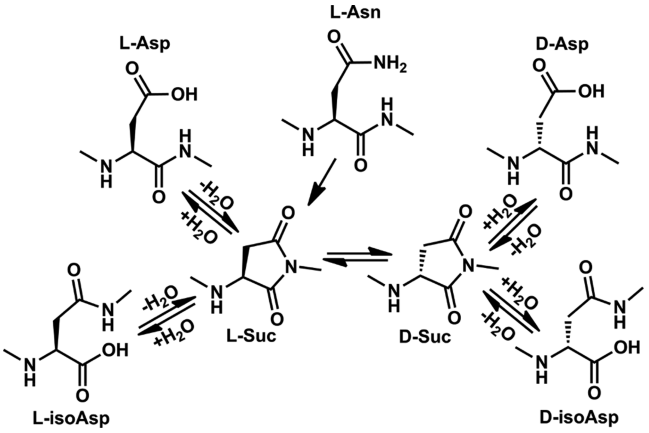
ABSTRACT: Deamidation of asparagine is a spontaneous and irreversible post-translational modification associated with a growing list of human diseases. While pervasive, deamidation is often overlooked because it represents a relatively minor chemical change. Structural and functional characterization of this modification is complicated because deamidation of asparagine yields four isomeric forms of Asp. Herein, radical directed dissociation (RDD), in conjunction with mass spectrometry, is used to identify and quantify all four isomers in a series of model peptides that were subjected to various deamidation conditions. Although primary sequence significantly influences the rate of deamidation, it has little impact on the relative proportions of the product isomers. Furthermore, the addition of ammonia can be used to increase the rate of deamidation without significantly perturbing isomer populations. Conversely, external factors such as buffer conditions and temperature alter product distributions but exhibit less dramatic effects on the deamidation rate. Strikingly, the common laboratory and biologically significant bicarbonate buffer is found to strongly promote racemization, yielding increased amounts of D-Asp and D-isoAsp. These outcomes following deamidation have broad implications in human aging and should be considered during the development of protein-based therapeutics.



Post-translational modifications (PTMs) behave as powerful regulatory elements that often define protein function through covalent modification. Because PTMs profoundly affect protein behavior, they are typically tightly controlled, and dysregulation is strongly associated with numerous diseases.[1] Deamidation is an exception among PTMs due to its nonenzymatic and spontaneous nature. Consequently, it is both unregulated and irreversible. The inevitable nature and predictable rate of this modification prompted the idea that deamidation may have been engineered as a molecular clock that signals protein aging and turnover.[2] Although there are select examples of deamidation serving functional roles, it is generally considered to represent degradation[3] and is associated with a growing list of age-related, neurological, aggregation-prone, and autoimmune diseases.[4]

While the implications of asparagine deamidation are becoming increasingly apparent, the specific structural outcomes have not been well-defined because the reaction yields four isomeric products: L-Asp, D-Asp, L-isoAsp, and D-isoAsp (see Scheme 1). All deamidation pathways introduce a negatively charged side chain under physiological conditions, but each of the four isomers is additionally likely to impact structure and function in distinct ways. Although L-Asp and D-Asp differ by only a single chiral center (thus making peptide epimers when present in pairs that otherwise have the same sequence), D-amino acids are known to have a profound impact on peptide function. For instance, the D-Ala epimer of the peptide opioid dermorphin (YAFGYPS) is ~1000 times more potent than morphine, while the L-Ala epimer is biologically inactive.[5] Unlike dermorphin, which is enzymatically modified in tree frogs (*Phyllomedusa sauvagei*), D-amino acids accumulate

Scheme 1. Deamidation Proceeds via an Intramolecular Nucleophilic Attack[a]



[a]The succinimide (Suc) intermediate is prone to racemization and hydrolysis yielding four Asp isomers.

Received: August 9, 2017
Accepted: October 6, 2017
Published: October 6, 2017

DOI: 10.1021/acschembio.7b00686
ACS Chem. Biol. 2017, 12, 2875–2882

in human proteins due to aging. Recently, racemization was reported to be the most abundant PTM in aged lens proteins.[6] Furthermore, D-Ser epimers have been shown to contribute to the toxicity of β-amyloid *in vivo*.[7] In addition to yielding racemized Asp, deamidation produces isoAsp, which incorporates a methylene group into the peptide backbone (Scheme 1). Isoaspartic acids are associated with protein aggregation,[8,9] most notably in the lens crystallin proteins where isomerization may induce aggregation[10] and promote cataract formation[11,12] and in β-amyloid peptides linked to Alzheimer's disease.[8,13,14] Additionally, isoaspartic acids are known to trigger autoimmune responses,[15] which may be involved in diseases such as multiple sclerosis.[16] Importantly, the B cell response triggered by isoAsp peptides from cytochrome *c* has been reported to be cross-reactive, targeting both the isoAsp peptides and the native protein in mice.[17] Low levels of deamidation may, therefore, trigger the removal of the entire protein population.

As a spontaneous process, the importance of deamidation extends outside the cell. Deamidation is a source of heterogeneity in recombinant protein production.[18] These structural changes have been shown to alter protein folding transitions[19] and may contribute to protein aggregation.[20] Degradation can continue during storage,[4] making deamidation an important consideration for therapeutic formulations.[21] Protein-based bioconjugates represent an increasingly popular choice for pharmaceuticals,[22,23] accentuating the need for better understanding of effective protein lifetime and degradation byproducts. For example, perturbations to complementarity-determining regions (CDRs) of antibodies can drastically reduce antigen binding efficacy,[24] as demonstrated by deactivation of monoclonal immunoglobulin γ2 following a single isomerization.[25] Furthermore, deamidation is facilitated by the solvent exposed and flexible nature of CDRs.

Under physiological conditions, deamidation proceeds through a succinimide intermediate that is formed as the backbone nitrogen (on the C-terminal side) attacks the side chain carboxyl group of asparagine[26] (Scheme 1). Therefore, peptide flexibility[27] and the amino acid on the C-terminal side of asparagine strongly influence the deamidation rate.[2] Catalysis by ammonia and increased temperature can accelerate deamidation.[28] Once formed, the asymmetric succinimide ring can be hydrolyzed to yield Asp or isoAsp with the isoAsp products being favored at a ratio of about 3:1.[26] The ring is prone to racemization prior to hydrolysis, and the resulting products include both the L- and D-stereoisomers of Asp and isoAsp. Asp residues are also susceptible to succinimide ring formation, ultimately yielding the same four products, but this reaction is significantly slower.

Despite the ease in detecting deamidation (which results in a 0.984 Da mass shift) via mass spectrometry (MS), high-throughput analysis of the four isomers generated by deamidation remains challenging.[29] Most chromatographic methods are limited to reporting the ratio of the Asp/isoAsp, although some isomeric peptides have been fully resolved via ultraperformance liquid chromatography (UPLC).[12] Additionally, ion mobility MS (IMS-MS) has been successfully employed to characterize peptide epimers[30] and β-amyloid isomers.[31] Mass spectrometry-based dissociation techniques can be used to detect isoAsp residues based on diagnostic c- and z- fragment mass shifts generated by electron capture dissociation (ECD),[32] and electron transfer dissociation (ETD).[33] Like ECD and ETD, radical directed dissociation (RDD) is a gas-phase radical-based dissociation technique, but RDD differs by generating radicals via site-specific photodissociation.[34] Radical migration facilitates cleavage of the peptide backbone as well as amino acid side chains.[35] Importantly, these radical migration pathways are structurally sensitive.[36] RDD has previously been used to distinguish peptide epimers based on fragmentation patterns[37] and identify Asp isomers via tandem LC-MS.[38] Despite these recent advances, there are few cases where deamidation has been systematically studied, likely due to time constraints and difficulty with distinguishing and quantifying the products.

Presented herein is a systematic evaluation of the four Asp isomers generated via deamidation, with a focus on the influence of primary peptide sequence, side chain chemistry, and solvent conditions. Short peptides were selected to test deamidation in the absence of confounding factors such as higher-order structure. We analyzed a series of hexapeptides that vary in sequence by a single amino acid C-terminal to asparagine. The sequence variations include glycine, which is side-chain deficient, alanine, which is apolar and has a minimal steric footprint, serine, which is polar but sterically unimposing, and finally histidine, which is polar, basic, and bulky. The peptides were deamidated under physiological, catalytic buffer, and high heat conditions. By detecting and quantitating all four isomers, the trends associated with these deamidation conditions can be identified. Importantly, D-asp and D-isoAsp were detected in appreciable amounts under all conditions and were found to significantly increase when deamidation was carried out in bicarbonate buffer or under elevated temperature conditions.

RESULTS AND DISCUSSION

Mass Spectrometry. All experiments were conducted with a series of synthetic peptides that varied in sequence by a single amino acid, 4IB-VKLNXG (where X = G, H, S, A and 4IB = *para*-iodobenzoic acid). The half-lives for these peptides at 37 °C in 50 mM Tris at pH 7.8 are 1.4, 6.8, 41, and 73 days, respectively. Based on related experiments conducted previously,[2] these sequences should be among the fastest to deamidate while still offering a variety in side chain chemistry. In addition, 4IB was added to each N-terminus to provide a photocleavable radical precursor for RDD experiments (see Supporting Information for structures). Tris has long been among the buffers of choice to study deamidation *in vitro* due to its modest influence on deamidation.[39]

Following sufficient incubation to deamidate a majority of the sample, peptides were separated and analyzed via LCMS. Typical results are shown in Figure 1a, which depicts the ion chromatogram (extracted from full MS scans for the m/z of the intact deamidated peptide) for 4IB-VKLDSG after incubation for 6 months. Only two peaks are apparent, though four Asp isomers could be generated by the pathways illustrated in Scheme 1. Given that the structural differences between L-Asp and D-Asp or L-isoAsp and D-isoAsp are quite subtle, it is likely that isomers have coeluted. Tandem MS can provide additional information on this possibility in the form of fragmentation profiles.

RDD tandem MS results are shown in Figure 1b–d for locations denoted 1–4, respectively, in Figure 1a. In RDD experiments, a radical is generated photolytically, and the product is subjected to collisional activation. Migration of the radical is structurally sensitive, leading to observation of distinct fragmentation patterns for isomers. Indeed, close examination of the spectra shown in Figure 1b–d reveals notable differences.

DOI: 10.1021/acschembio.7b00686
ACS Chem. Biol. 2017, 12, 2875–2882

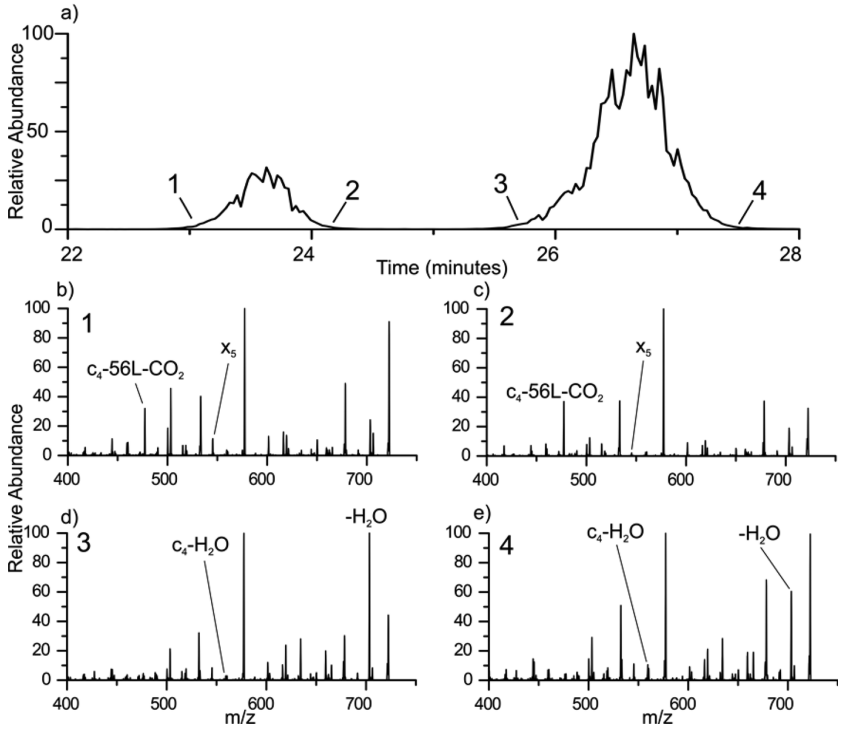


Figure 1. (a) LCMS chromatogram of the 4IB-VKLDSG deamidation products, which yields only two chromatographic peaks. (b−e) RDD tandem mass spectra on $[4IB\text{-}VKLDSG + H]^{1+}$ from the leading and trailing edges of the peaks in panel a show differences in fragment intensities that reveal coeluting isomers within both peaks.

The standard method for quantifying such differences is to calculate an R_{isomer} value, which is obtained from the ratios of product ions changing the most between two spectra (see Supporting Information for additional details).[40] R_{isomer} values for all possible pairs from Figure 1b,c were calculated, which identified the x_5 and c_4-56L-CO_2 fragments as those undergoing the greatest relative changes. These fragments yield an R_{isomer} value of 6.3, which is well above the threshold for isomer identification (>2.4). A similar analysis revealed an R_{isomer} value of 6.1 for the diagnostic fragments labeled in Figure 1d,e. Fully annotated spectra are available in the Supporting Information.

The c_4-56L-CO_2 fragment corresponds to a c_4 backbone fragment that has additionally lost CO_2 and 56 Da from the leucine side chain (i.e., 56L). Such multiple dissociation events and side chain losses are common in RDD.[35] Predicting the fragments that will yield the maximum R_{isomer} values is not trivial because changes in fragment abundances are due to differences in three-dimensional structure (not sequence). Collision-induced dissociation (CID) was also used to generate fragmentation spectra for all peptides examined herein, but as a less structurally sensitive method, CID usually yields lower R_{isomer} values. The identity of each isomer in Figure 1a can be determined by comparing spectra from the leading and trailing edge of each peak to synthetic standards. The spectrum in Figure 1b best matches that from D-Asp, while Figure 1c matches L-Asp. The dominant isomer represented at each labeled point in Figure 1a therefore corresponds to 1 = D-Asp, 2 = L-Asp, 3 = D-isoAsp, and 4 = L-isoAsp. A full list of diagnostic peaks and R_{isomer} values used for quantitation is provided in the Supporting Information.

Although the R_{isomer} values readily confirm coelution, they do not allow quantitation of each isomer. Results from the synthetic standards for L-Asp and D-Asp isomers of 4IB-VKLDSG are shown in Figure 2a,b, where again the x_5 and c_4-56L-CO_2 abundances differ significantly between the spectra. By plotting the difference/sum of the two diagnostic peaks as a function of the percent L-Asp, the calibration curve shown in Figure 2d was generated. Figure 2c shows the averaged fragmentation pattern obtained from the entire LC peak at ∼23.5 min in Figure 1. While similar to both standards, Figure 2c is not a perfect match to either panel a or panel b of Figure 2 because it contains contributions from both isomers. In order to quantify the amount of each isomer present, the data from Figure 2c was mapped onto the calibration curve shown in Figure 2d. The LC peak at 23.5 min in Figure 1 contains 88% L-Asp and 12% D-Asp.

This methodology was repeated using both RDD and CID for each peptide. Calibration curves were generated using the fragmentation method that afforded the best isomer discrimination. Interestingly, 4IB-VKLNAG separates into three fully resolved peaks, representing D-Asp, L-Asp, and L-/D-isoAsp. Quantitation via peak area matched the results derived from the RDD calibration curve for the ratio of L-Asp and D-Asp to within 0.5% agreement (see Supporting Information), offering independent confirmation of our approach.

The results obtained in Tris at pH 7.8 for all four peptide sequences are summarized in Figure 3. Despite having considerably different half-lives and side chain chemistry, the results from all four peptides exhibit similar trends. L-isoAsp is generated at 68.2% on average and is confirmed to be the dominant product for each peptide sequence, in agreement with previous findings.[41] In comparison, L-Asp represents only 20.3% of the total deamidation products on average. A similar preference for the iso-variant is observed between D-isoAsp and D-Asp, with D-isoAsp present at 9.1% on average compared to 2.4% for D-Asp. The paucity of D-Asp is easily rationalized because its formation requires both racemization of the succinimide ring and hydrolysis to open the ring via the less

DOI: 10.1021/acschembio.7b00686
ACS Chem. Biol. 2017, 12, 2875−2882

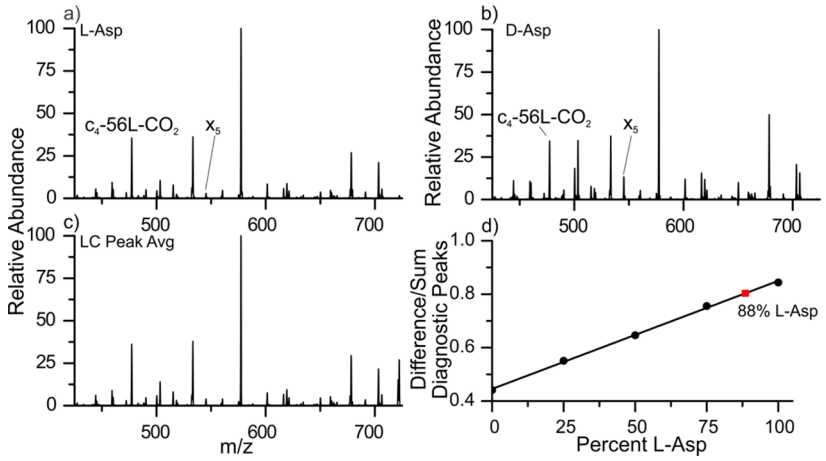


Figure 2. (a,b) Standards of the L-Asp and D-Asp isomers of 4IB-VKLDSG are distinguished by diagnostic fragment peaks. (c) The average fragmentation abundance for the LC peak that contains both isomers. (d) A calibration curve generated from purified standards reveals the LC peak contains 88% L-Asp (red square).

favorable mechanism. On the other hand, the abundance of D-isoAsp is significant and illustrates that racemization during deamidation should not be ignored.

Although deamidation always results in the mutation of asparagine to aspartic acid, the observation that ∼80% of the total deamidation products represent unnatural amino acids may explain the widespread immunogenic responses associated with deamidation *in vivo*.[42−44] For the same reason, deamidation in protein therapeutics is likely to have undesirable effects ranging from loss of activity to altered immunogenicity.[44,45] The high abundance of D-isoAsp may also be cause for a re-examination of previous conclusions discounting its importance. For example, the presumed-to-be negligible abundance of D-isoAsp was previously used to rationalize the substrate specificity of the repair enzyme, protein L-isoaspartate methyltransferase (PIMT), which can bind L-isoAsp and D-Asp but not D-isoAsp residues.[9] Given that D-Asp is significantly less abundant than D-isoAsp, PIMT specificity is likely due to other factors. One potential explanation is that PIMT requires the α-nitrogen atom to be positioned "behind" the methyl-accepting carboxyl group as is the case for L-isoAsp and D-Asp, but not L-Asp or D-isoAsp.[41]

Buffer Conditions. The influence of buffer, pH, temperature, and ammonia (a known catalyst)[28] were explored, and the results are summarized in Figure 4. To simplify comparison, the relative abundances of each isomer are plotted together for

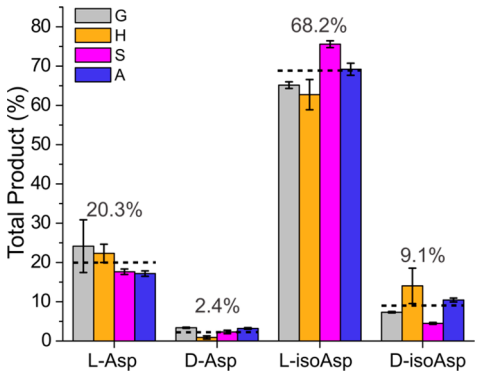


Figure 3. Deamidation products at 37 °C in 50 mM Tris at pH 7.8. Averages are indicated with dashed lines.

all conditions that were examined. The first grouping for each isomer represents data acquired at 37 °C in 50 mM Tris at pH 7.8 and is derived from the results shown in Figure 3 (replotted for easy comparison). For all remaining experiments ammonia was added (indicated by the green box) as ammonium hydroxide to a final concentration of 100 mM ammonia in Tris and CHES at each pH or as 100 mM ammonium bicarbonate for the carbonate buffer. Ammonia has been reported to accelerate deamidation through general base catalysis,[28] but its influence on deamidation products has not been previously determined. Although rates are accelerated (as discussed in greater detail below), the addition of ammonia and a slight increase to pH 8.8 do not significantly influence the distribution of Asp isomers generated by deamidation in Tris buffer (compare Figure 4, Tris 7.8 and Tris 8.8).

Results obtained in CHES buffer at pH 8.8 and 9.8 are also shown in Figure 4. Although CHES buffer and Tris are chemically dissimilar and have pK_a's of 9.5 and 8.1, respectively,[46] there is little influence on the overall distribution of Asp isomers produced by the two buffers. Furthermore, increasing the pH to 9.8 does not have a significant impact on the results, though minor variations in product abundance are noted. Intracellular pH can vary by over 3 pH units, ranging from 4.7 to 8.0.[47] Our data indicate that even the most basic intracellular conditions are unlikely to strongly influence succinimide-mediated deamidation products.

The effect of heating was explored by raising the temperature to 80 °C in CHES at pH 9.8 (these results are marked with an asterisk in Figure 4). Increasing the temperature impacts the resulting isomer distribution by decreasing the amounts of L-Asp and L-isoAsp while increasing the proportion D-Asp and D-isoAsp at the same time. These results suggest that higher temperature leads to greater racemization of the intermediate succinimide ring, leading to greater production of the D-isomers. After racemization, ring-opening again favors formation of the iso-products. Consequently, elevated temperatures used previously to accelerate deamidation *in vitro* may have been biased toward the production of the D-isomers.[28,48] Interestingly, the increase in D-Asp observed at elevated temperatures may have prompted an evolutionary adaptation in the hyperthermophile *Pyrococcus furiosus*, which expresses a variant of PIMT that repairs D-Asp containing peptides with

DOI: 10.1021/acschembio.7b00686
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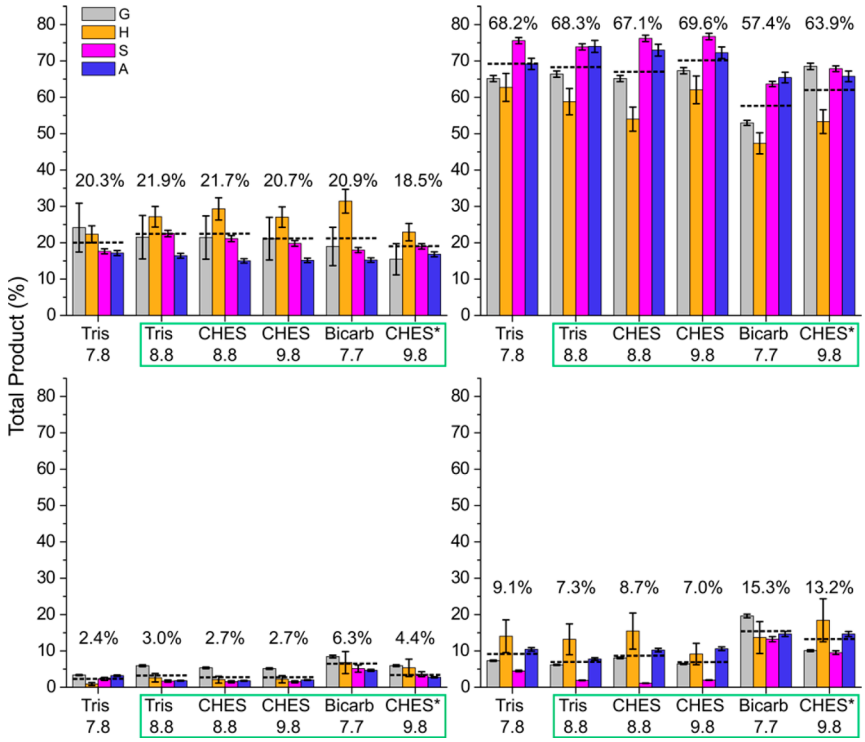

Figure 4. Total product outcomes from 6 different buffer conditions. Averages are depicted with horizontal bars. Green box indicates ammonia catalysis. The asterisk indicates 80 °C.

120-fold higher affinity relative to the human enzyme.[49] D-isoAsp, however, is still not a substrate for this variant.

Experiments with bicarbonate buffer at pH 7.7 also revealed altered isomer product distributions. Although the amount of L-Asp generated is comparable to other buffers, the production of L-isoAsp is significantly reduced. This observation suggests that ring-opening to produce L-isoAsp is specifically disfavored by the presence of bicarbonate. The isomer abundance that would typically end up in the L-isoAsp channel is redirected to the D-isomers, which are both more abundant relative to other buffer conditions. These results from bicarbonate differ from those obtained at 80 °C because the increased yield of D-isomers derives from only L-isoAsp rather than from a diminution of both L-isomers. The enhanced racemization observed in bicarbonate is of particular interest because bicarbonate has been the buffer of choice for many deamidation and digestion studies.[50−52] Bicarbonate also plays a prominent role in the native blood-buffer system.[53] It is surprising that bicarbonate buffer yields the highest amount of racemization despite having the lowest initial pH (7.7). Carboxylic acids have been reported to stabilize deamidation transition states. We attribute the increase in racemization to noncovalent interactions with bicarbonate that disfavor formation of L-isoAsp.

Though spontaneous and pervasive, deamidation is typically slow under physiological conditions, which has deterred large-scale comprehensive studies and favored examination of sequences that deamidate quickly. Evaluation of deamidation can also be complicated by spontaneous degradation for certain peptide sequences.[28,54] These limitations could potentially be overcome if the rate of deamidation could be accelerated without biasing the products or favoring other degradation pathways. Our results suggest that both Tris and CHES based buffer systems can be used with ammonia as a catalyst over a wide pH range without significantly influencing the distribution of Asp isomers that will be formed. However, caution should be taken if elevated temperature, bicarbonate, or other untested buffers are to be used. Although the ratio of (total isoaspartic acids)/(total aspartic acids) has long been the metric of choice for deamidation studies due to the relative ease of analysis, our data reveal that the overall product distribution can shift significantly without changing the isoAsp/Asp ratio. For instance, the isoAsp/Asp ratio for CHES 9.8 at 37 °C is 3.3 while the ratio at 80 °C is 3.4 despite the noted increase in D-isomers. Chirally distinct isomers are likely to have different biological impacts, emphasizing the importance of quantitating all four isomers.

Rate Enhancement. The degree of rate enhancement relative to Tris (pH 7.8, 37 °C) is shown as a fold increase in Figure 5 for each buffer and peptide sequence. On average, Tris 8.8 was found to be 17× faster than Tris 7.8, where potential acceleration derives from both increased pH and ammonia catalysis. Comparing CHES at 8.8 and 9.8 reveals that increasing the buffer by one pH unit yields only a modest increase in the rate, from 1.2× to 2.0× faster (relative to Tris 7.8). This observation suggests that the bulk of the acceleration between the Tris samples is due to the presence of ammonia. This rate enhancement, along with the modest effect on isomer product distributions shown above, suggests ammonia is a viable catalyst for deamidation studies.

The most dramatic average rate increase (81×) was observed by increasing the temperature to 80 °C in CHES. At this temperature, over three-quarters of 4IB-VKLNGG was deamidated within 30 min, making it difficult to determine the deamidation rate accurately. The reported rate increase for this peptide in Figure 5 is likely accompanied by significant uncertainty, though deamidation clearly occurs rapidly. Although elevated temperature appears to provide the greatest

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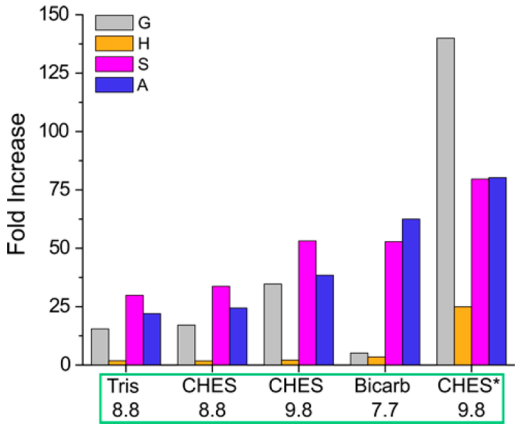

Figure 5. Deamidation rate increases relative to Tris 7.8 for each accelerated buffer condition. The green box indicates ammonia catalysis. The asterisk indicates 80 °C.

increase in deamidation rate, higher temperature also influences the product distribution, and yields increased racemization.

Strikingly, the rate of acceleration varies greatly among different peptide sequences. Most notably, the histidine-containing peptide is least affected by the addition of ammonia. This result is particularly interesting because peptides with asparagine followed by histidine have previously attracted attention due to their rapid deamidation rates despite the bulky nature of histidine. Ultimately, this rate enhancement has been attributed to the ability of the histidine side chain to act as a general base.[54] If true, the addition of ammonia, which also enhances the rate of deamidation by general base catalysis,[28] would not be expected to have a significant effect, in agreement with the results in Figure 5. Importantly, the histidine peptide does deamidate more quickly at increased temperature, which has little effect on acid/base chemistry but increases the energy available to the system. This observation further confirms that the basic side chain of histidine likely explains the lack of response to the addition of ammonia.

Curiously, the rate of deamidation does not increase as significantly with ammonia for 4IB-VKLNGG in bicarbonate, though increases for this peptide were comparable to the other nonhistidine residues in all other conditions. This result may suggest that carbonate interacts strongly with the backbone, inhibiting access to the required transition state. The absence of a side chain for the glycine peptide may increase interactions between carbonate and the backbone.

CONCLUSIONS

Although deamidation yields similar product trends among vastly different buffer conditions and peptide sequences, it is noticeably sensitive to specific extrinsic factors. Both bicarbonate buffer and high temperature strongly promote racemization, while increasing pH from 7.8−9.8 has a surprisingly limited effect. Importantly, we have observed that D-isomers, which are often ignored, are generated in appreciable quantities under all conditions. These results establish the innate deamidation tendencies for all four Asp products and demonstrate that isomer distributions are susceptible to external influences. Furthermore, this methodology should be able to quantify isomers for many classes of peptides, including isomers identified in proteomics experiments targeting long-lived proteins relevant to aging, human disease, and biological therapeutics. Understanding the products of deamidation, and the factors that alter them should facilitate more reliable prediction and control of degradation products. This information may become integral for engineering proteins that minimize the detrimental effects of deamidation and isomerization.

METHODS

Materials. Organic solvents and reagents were purchased from Fisher Scientific or Acros Organics and used without further purification. Water was purified to 18.2 MΩ using a Millipore 147. FMOC-protected amino acids and Wang resins were purchased from Anaspec, Inc. or Chem-Impex International.

Peptide Synthesis. Peptides were synthesized manually following an accelerated FMOC-protected solid-phase peptide synthesis protocol.[55] *Para*-iodobenzoic acid (4IB) was attached to the N-terminus of the nascent polypeptide during synthesis. Following synthesis, peptides were purified using a Phenomenex Jupiter Proteo C12 4 μm 90 Å 250 mm × 4.6 mm column. Purified peptides were stored frozen in 50/50 acetonitrile/water (v/v).

Deamidation. Peptides were deamidated in various conditions to explore the impact of buffer, pH, ammonia catalysis, and temperature. Tris (50 mM, pH 7.8) at 37 °C results are quantitated and used as reference values for subsequent comparisons, which include 50 mM Tris, pH 8.8, + 100 mM NH_3 at 37 °C, 50 mM CHES, pH 8.8, + 100 mM NH_3 at 37 °C, 50 mM CHES, pH 9.8, + 100 mM NH_3 at 37 °C, 100 mM ammonium bicarbonate at 37 °C, and 50 mM CHES + 100 mM NH_3 at 80 °C. For the Tris and CHES buffers at pH 8.8 and 9.8, ammonium hydroxide was added at 381 and 128 mM, respectively, to yield a final concentration of 100 mM unprotonated ammonia.

Analysis. Following deamidation, peptides were analyzed via LCMS. An Agilent 1100 binary pump was used with a 5 μm 100 Å C5 50 mm × 2 mm column (Phenomenex) interfaced to a Thermo Fisher Scientific LTQ mass spectrometer with a standard electrospray ionization source. Deamidated peptides were eluted with an isocratic mixture of 18% acetonitrile and 82% water with 0.1% formic acid (v/v). Synthetic standards were prepared as ∼10 μM samples in 49.5/49.5/1 methanol/water/acetic acid (v/v) and infused into a modified LTQ linear ion trap using the standard electrospray ionization source. The LTQ was modified with a quartz window to allow fourth harmonic (266 nm) laser pulses from a Nd:YAG laser to irradiate the trapped ion cloud, which allows for photoinitiated radical directed dissociation.[56]

R_{isomer} Values. Isomers are distinguished based on MS^n fragmentation spectra. To quantitatively evaluate the differences between two spectra, R_{isomer} values are calculated using eq 1 where R_1 and R_2 refer to the ratios of a pair of fragment ions that vary the most in abundance between two different MS^n scans.

$$R_{isomer} = \frac{R_1}{R_2} \quad (1)$$

Identical fragmentation patterns result in R_{isomer} values of 1, thereby indicating no discrimination, while larger values reflect a higher degree of discrimination. Values >2.4 obtained via LCMS are indicative of analytes that can be distinguished based on fragmentation patterns.[38]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00686.

Additional LCMS chromatograms, RDD calibration curves, peptide structures, and R_{isomer} details (PDF)

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DOI: 10.1021/acschembio.7b00686
ACS Chem. Biol. 2017, 12, 2875−2882

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge funding from the National Institutes of Health (Grant R01GM107099).

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