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Deuteration protects asparagine residues against racemization

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Abstract

Racemization in proteins and peptides at sites of L-asparaginyl and L-aspartyl residues contributes to their spontaneous degradation, especially in the biological aging process. Amino acid racemization involves deprotonation of the alpha carbon and replacement of the proton in the opposite stereoconfiguration; this reaction is much faster for aspartate/asparagine than for other amino acids because these residues form a succinimide ring in which resonance stabilizes the carbanion resulting from proton loss. To determine if the replacement of the hydrogen atom on the alpha carbon with a deuterium atom might decrease the rate of racemization and thus stabilize polypeptides, we synthesized a hexapeptide, VYPNGA, in which the three carbon-bound protons in the asparaginyl residue were replaced with deuterium atoms. Upon incubation of this peptide in pH 7.4 buffer at 37 °C, we found that the rate of deamidation via the succinimide intermediate was unchanged by the presence of the deuterium atoms. However, the accumulation of the D-aspartyl and D-isoaspartyl forms resulting from racemization and hydrolysis of the succinimide was decreased more than five-fold in the deuterated peptide over a 20 day incubation at physiological temperature and pH. Additionally, we found that the succinimide intermediate arising from the degradation of the deuterated asparaginyl peptide was slightly less likely to open to the isoaspartyl configuration than was the protonated succinimide. These findings suggest that the kinetic isotope effect resulting from the presence of deuteriums in asparagine residues can limit the accumulation of at least some of the degradation products that arise as peptides and proteins age.

Keywords

Asparagine; Deamidation; Deuterium; Racemization

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Compliance with ethical standards

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Introduction

One of the most prevalent spontaneous chemical reactions that lead to the degradation of proteins and peptides is the racemization of L-amino acids. The rate of racemization differs with each amino acid residue, with aspartic acid and asparagine being particularly susceptible (Bada 1983; Ritz-Timme and Collins 2002; Ohtani et al. 2004; Truscott and Friedrich 2016). Racemization at aspartyl and asparaginyl residues could contribute not only to the loss of function of protein pharmaceuticals during storage and use (Manning et al. 1989; Zhang et al. 2011) but for the loss of protein function in organisms, particularly of the long lived species in eye lens (Hooi and Truscott 2011), teeth (Ohtani and Yamamoto 2010), and red blood cells (Brunauer and Clarke 1986). Racemized derivatives arising from L-aspartate and L-asparagine accumulate with age and reach about 8% of the total protein aspartate/asparagine content in the proteins of human tooth enamel by 60 years of age (Helfman and Bada 1975), and 10% of the total aspartate/asparagine residues in human eye lens crystallins by 70 years of age (Masters et al. 1977; Hooi and Truscott 2011). Protein D-aspartyl residues can be recognized by the protein repair L-isoaspartate-(D-aspartate) *O*-methyltransferase, an enzyme present in most organisms and cell types that limits L-isoaspartate and presumably D-aspartate accumulation (Lowenson and Clarke 1992; Patananan et al. 2014). However this reaction can lead to the formation of D-isoaspartyl residues and can thus enhance the accumulation of racemized residues (Lowenson and Clarke 1992; Young et al. 2005).

D-aspartyl derivatives also accumulate in proteins that are prone to aggregate in several human pathologies. Proteins from brunescent cataracts have about twice the increase in the D-Asp/L-Asp ratio in hydrolysates compared to non-cataractous lenses of comparable age (Masters et al. 1977; Hooi et al. 2012). Additionally, the aspartyl and isoaspartyl residues in Alzheimer's disease brain neuritic plaque beta-amyloid have a D/L ratio of about 11% (Roher et al. 1993a,b; Inoue et al. 2014). The abundance of racemized aspartyl and isoaspartyl residues is considerably less in vascular β-amyloid from these brains, suggesting that the vascular amyloid aggregates are "younger" than the neuritic amyloid plaques (Roher et al. 1993a,b). These and other findings suggest that racemization of amino acid residues in long-lived proteins, particularly that arising from aspartyl and asparaginyl residues, might contribute to the loss of organ/tissue function with age and progression of diseases involving protein accumulation (reviewed in Ritz-Timme and Collins 2002).

The mechanism by which aspartate and asparagine residues are particularly sensitive to racemization involves the reaction between the side chain carbonyl group with the peptide bond nitrogen of the following residue to produce a succinimide ring (Fig. 1a, Geiger and Clarke 1987). Racemization occurs after the loss of a proton from the alpha carbon of these species to form a resonance-stabilized planar carbanion that can be reprotonated from either side to give a mixture of L- and D-succinimide (Fig. 1b). The decrease in the pKa of the proton on the alpha carbon on the succinimide residue has been supported by *ab initio* studies (Radkiewicz et al. 1996). The succinimide is also subject to hydrolysis at either of its two carbonyl carbons. Hydrolysis at the carbonyl that was formerly in the peptide's backbone occurs (at least in peptides) about 75% of the time, resulting in an L- or D-isoaspartyl residue, in which the peptide backbone now goes through the former side-chain

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of the amino acid (Fig. 1a, Geiger and Clarke 1987; Stephenson and Clarke 1989; Manning et al. 1989). The rest of the time, hydrolysis occurs at the former side-chain carbonyl, generating either an L- or D-aspartyl residue. Recent work has also suggested that racemization can occur via a radical mechanism (Tambo et al. 2013).

We have been interested in investigating methods by which the potentially detrimental accumulation of racemized residues in proteins in the cell as well as in therapeutic peptide and protein preparations could be diminished. Classical kinetic isotope effects (KIE) occur when an atom is replaced by its heavy isotope, such as ^{2}H or ^{13}C. For non-enzymatic reactions, the deuterium KIE is typically between 2–9 (i.e. cleavage of a carbon-deuterium bond is 2–9 times slower than an equivalent carbon-proton bond) (Westheimer 1961). It was proposed that deuteration at damage-vulnerable sites of biomolecules could slow down degradation if hydrogen abstraction is the rate limiting step (Shchepinov 2007; Shchepinov et al. 2010). Recently, it has been observed that deuteration of specific sites in polyunsaturated fatty acids (PUFA) inhibits lipid peroxidation, and supplementing *Saccharomyces cerevisiae* and cultured mammalian cells with deuterated PUFA protects these cells from oxidative stress (Hill et al. 2012; Andreyev et al. 2015). This protective effect is seen even when as little as 20% of the PUFAs have a single deuterated bis-allytic position (Hill et al. 2012). Thus, replacing the protons bound to the side chain methylene and alpha carbons of an L-asparaginyl residue in a polypeptide with deuterium atoms could potentially change the rate at which this residue undergoes deamidation via succinimide formation, the rate at which the L-succinimide racemizes to the D-succinimide form, and the tendency of the succinimide to open to the isoaspartyl form rather than the aspartyl form. A change in the rate of succinimide formation would result if these deuterium atoms change the nucleophilicity of the backbone nitrogen and/or the electrophilicity of the gamma carbonyl carbon involved the nucleophilic attack. Since racemization of the succinimide proceeds via the loss of the proton on the alpha carbon, replacing this hydrogen with a deuterium atom should decrease the concentration of the racemization-prone carbanion (Radkiewicz et al. 1996). It is also possible that deuterium atoms on the beta carbon of the side chain could alter the resonance forms provided by the ring structure. Finally, it is currently unclear why the carbonyl carbon in the position that was formerly the alpha carbonyl of the asparaginyl/aspartyl residue is more susceptible to hydrolysis than the former side chain carbonyl carbon, but deuteriums replacing the methylene group hydrogens in the succinimide ring could potentially alter the susceptibility of the two carbonyl groups by altering the resonance, or for steric reasons.

To determine how the presence of deuteriums might affect the deamidation, racemization, and isoaspartyl formation of an asparaginyl residue, we generated peptides with the sequence L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala, in which one peptide was made in the usual way and another made using an asparagine derivative where the hydrogens on the alpha carbon and the side chain methylene group were substituted with deuterium atoms (Fig. 1c). This amino acid sequence was derived from a degradation prone region of human adrenocorticotropic hormone (Aswad 1984a; Murray and Clarke 1984). This sequence was also chosen because the asparaginyl-, aspartyl-, and isoaspartyl-forms of this peptide can be separated and quantified by reverse-phase HPLC, and because the rates of succinimide formation and epimerization of the protonated peptide have been previously measured

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(Geiger and Clarke 1987; Stephenson and Clarke 1989). Although we found no effect of deuteration to the rate of succinimide formation, and only a small effect on the rate of succinimide hydrolysis, we detected a five-fold decreased rate of D-aspartate formation, suggesting that deuteration can protect against racemization.

Materials and methods

Preparation of a protected deuterated asparagine derivative for peptide synthesis

d3-Fmoc-L-Asn(Trt)-OH (Fig. 1d) was prepared from d3-L-Asn(Trt)-OH 0.5 H_2O and N-(9-fluorenylmethoxycarbonyloxy)succinimide as white crystals (0.125 g, 77%) following the general procedure described in the literature (Albericio 2000). **M.p**. 208–210 °C. **TLC**: R_f 0.35 ($CHCl_3$/MeOH/AcOH 94:5:1). Both the ^1H and ^{13}C NMR spectra for d3-Fmoc-L-Asn(Trt)-OH were recorded in $CDCl_3$ on a Bruker AV-400 instrument at room temperature. Chemical shifts are reported in ppm relative to TMS with the residual solvent peak used as an internal standard (references for DMSO-d_6: ^1H 2.50 ppm, ^{13}C 39.43 ppm). The ^{13}C NMR spectrum was recorded with complete proton decoupling at 100.6 MHz. **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 12.73 (br s, 1H), 8.64 (s, 1H), 7.90 (d, J = 7.4 Hz, 2H), 7.73 (d, J = 7.7 Hz, 2H), 7.63 (s, 1H), 7.47-7.11 (m, 19H), 4.44-4.11 (m, 3H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 173.1, 168.7, 155.7, 144.6 (×3), 143.7 (×2), 140.6 (×2), 128.5 (×6), 127.6 (×2), 127.3 (×6), 127.0 (×2), 126.3 (×3), 125.1 (×2), 120.0 (×2), 69.3, 65.6, 46.6.

Peptide synthesis

VYPNGA peptides were synthesized either with the deuterated Fmoc Asn derivative described above or with the standard Fmoc Asn derivative as trifluoroacetate salts by JPT Peptide Technologies, GmbH, Germany, with >95% purity, as determined by C18 reverse-phase HPLC with a linear gradient and detection at 220 nm. Mass spectrometric analyses using electrospray ionization confirmed the structure of both peptides with ions of m/z 623.3 $[M+H]^+$, 1244.6 [2M+H] for the deuterated peptide (calculated 622.68) and 620.3 $[M+H]^+$, 1240.6 $[2M+H]^+$ for the non-deuterated peptide (calculated 619.68).

In vitro peptide aging

Deuterated peptide VYPNGA and the protonated control peptide were dissolved in 0.1 M sodium phosphate, pH 7.44 at 23 °C. These solutions were sterilized by passing them through 0.2 μm pore syringe filters (Fisher), and 50 μL aliquots containing 40 nmol of peptide were placed in 200 μL PCR tubes, which were incubated at 37 °C for various times up to 20.6 days. At this temperature, the pH of the phosphate buffer is very close to 7.40. At the end of the incubations, 4.5 μL of 1 M hydrochloric acid was added to each tube, lowering the pH to about 4 to minimize any additional deamidation of the asparaginyl residue (Liu et al. 2016). These aged samples were then stored at −20 °C prior to analysis by HPLC.

Quantification of D-aspartic acid in peptide hydrolysates

First, a 2 to10 μL aliquot (1.3 to 7.3 nmol) was removed from the aged sample and dried in a small glass test tube. These tubes were placed in a hydrolysis vial and 300 μL of 6 M hydrochloric acid was added to the vial but not directly into the sample tubes. The vial was

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capped with a Teflon stopcock and subjected to three cycles of vacuum and nitrogen flushing before being sealed under vacuum using a PicoTag Work Station (Waters). This method allows only acid vapor to contact the peptide, minimizing contamination with exogenous aspartic acid. Hydrolysis proceeded at 110 °C for the relatively short time of 3 h, which is long enough to release most of the L- and D-aspartyl, isoaspartyl, succinimidyl, and asparaginyl residues in the peptides into free L- and D-aspartic acid, while minimizing acid-catalyzed racemization (Brunauer and Clarke 1986). L- and D-aspartate in the hydrolysates were then modified with o-phthalaldehyde and N-acetyl-L-cysteine to make fluorescent diastereomers and quantitated using reverse phase HPLC with fluorometric analysis (Aswad 1984b), as modified by Warmack et al. 2016.

Determination of racemization rate constants

To calculate the racemization rate constants, we modeled the degradation pathway using the rate constants determined by Geiger and Clarke (1987) for VYPNGA containing protonated asparagine in the following equations, where each k value has the units of min^{-1}:

L-Asn = Lasn−(Lasn*0.00034)

L-imide = Limide+(Lasn*0.00034)+(Lasp*0.0000099)+(Liso*0.0000099)+(Dimide*0.0002967)−(Limide*0.0002967)−(Limide*0.0039333)−(Limide*0.0010767)

L-isoAsp = Liso+(Limide*0.0039333)−(Liso*0.0000099)

L-Asp = Lasp+(Limide*0.0010767)−(Lasp*0.0000099)

D-imide = Dimide+(Dasp*0.0000125)+(Diso*0.0000125)+(Limide*0.0002967)−(Dimide*0.0002967)−(Dimide*0.0019333)−(Dimide*0.000625)

D-isoAsp = Diso+(Dimide*0.0019333)−(Diso*0.0000125)

D-Asp = Dasp+(Dimide*0.000625)−(Dasp*0.0000125)

At time zero, the L-asparaginyl-containing peptide is set to a quantity of 1.0, and all of the degradation products are considered to have a quantity of 0. The equations are then solved to give the quantities of the peptides present after one minute, and these quantities are then used to solve the peptides for the next minute. Using Microsoft Excel, these calculations were repeated until the amount of each peptide present after 20.6 days was determined.

Results and discussion

Replacing the hydrogen atoms of the asparaginyl residue in VYPNGA with deuterium atoms does not affect its rate of deamidation at pH 7.4 and 37 °C

Deuterated and non-deuterated VYPNGA were aged *in vitro* in 0.1 M sodium phosphate at physiological pH and temperature. The rate of deamidation of the asparaginyl residue was determined by separating the peptide from its degradation products by reverse phase HPLC using the approach of Geiger and Clarke (1987) and measuring the UV absorbance at 210 nm. We observed that the deuterated peptide with intact asparagine elutes at the same

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position in the buffer gradient as the protonated peptide (Fig. 2). Upon incubation at pH 7.4 and 37°C, the primary products of spontaneous deamidation of the deuterated asparaginyl residue, the L-isoaspartyl- and L-aspartyl-containing peptides, also elute at the same positions as the comparable protiated peptides (Fig. 2). The identity of the earlier eluting product of deamidation as the L-isoaspartyl-containing peptide is supported by its similar elution position to that determined by Geiger and Clarke (1987) for synthetic standards and the observation that it is approximately three-fold more abundant than the normal aspartyl-containing peptide eluting after the asparaginyl-containing peptide. This identification was confirmed for both the protiated and deuterated peptides by showing that only the early eluting peptide was a substrate for the recombinant human L-isoaspartate-(D-aspartate) *O*-methyltransferase (data not shown). Plotting the loss of the asparaginyl-containing peptide with time (Fig. 3) shows that the deamidation of the deuterated asparaginyl residue ($t_{1/2}$ = 1.39 days) proceeds at approximately the same rate as does the protiated residue ($t_{1/2}$ = 1.35 days), and this rate is very similar to values that have been previously reported for the protiated peptide ($t_{1/2}$ = 1.42 days, Geiger and Clarke 1987; $t_{1/2}$ = 1.14 days, Stephenson and Clarke 1989). This suggests that the nucleophilicity of the glycyl residue's backbone nitrogen and the electrophilicity of the asparaginyl residue's side-chain carbonyl carbon are not affected by the deuterium atoms in such a way that the rate of the nucleophilic reaction that forms the succinimide is changed.

The presence of deuterium atoms slightly alters the relative rates of hydrolysis at the succinimide intermediate's two carbonyl carbons

Both isoaspartyl- and normal aspartyl-containing peptides arise from spontaneous deamidation of the deuterated asparaginyl residue upon incubation at pH 7.4 and 37°C (Fig. 2), suggesting that the deamidation reaction proceeds via a succinimide intermediate as has been previously observed with protiated VYPNGA (Geiger and Clarke 1987). The succinimide ring contains two carbonyl carbons, both of which are sites of spontaneous hydrolysis; the half-life of this intermediate at physiological pH and temperature has previously been shown to be about 2.3 hours (Geiger and Clarke 1987). As is seen in Fig. 2, hydrolysis of the succinimide ring favors creation of isoaspartate over normal aspartate in the product. When the quantities of isoaspartyl- and normal aspartyl-containing peptides were measured in the HPLC experiments of all samples that had been aged at 37 °C for at least 24 h, we found that the average relative amount of isoaspartyl-containing peptide (isoAsp/(isoAsp + Asp)) is 0.793 (n = 18; standard deviation 0.009) for the protiated deamidation products and 0.770 (n = 17; standard deviation = 0.012) for the deuterated deamidation products. Statistical analysis with the Student t-test shows that these values are significantly different ($p = 4.2 \times 10^{-7}$). Thus, the succinimides generated from both of our peptides hydrolyze to form isoaspartyl residues more frequently than normal aspartyl residues as was previously reported for the protiated peptide ((L-isoAsp/(L-isoAsp + L-Asp) = 0.785; Geiger and Clarke 1987), but we now find that the presence of deuteriums rather than hydrogens on the succinimide intermediate improves hydrolysis at the former side chain carbonyl carbon, increasing the production of the normal aspartyl-containing peptide upon hydrolysis of the ring by about 2.3%.

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The presence of deuteriums on the asparaginyl residue significantly decreases its rate of racemization

To investigate the effect of asparagine residue deuteration on racemization, the peptides were aged at 37 °C and pH 7.4 as described above, and the quantity of D-aspartate determined following acid hydrolysis. While breaking the peptide bonds to release free amino acids, acid hydrolysis also converts asparaginyl, succinimidyl, and isoaspartyl residues into free aspartic acid. As can be seen in Fig. 4, the peptide containing the deuterated asparaginyl residue is much more resistant to racemization than is the protiated peptide. We modeled the accumulation of D-Asx residues (D-Asp, D-isoAsp, and D-succinimide) using the rate constants obtained by Geiger and Clarke (1987) as described in the "Materials and methods" and then varying the rate constant of succinimide racemization. Using least squares analysis to compare the modeled D-Asx accumulation to our experimentally measured values, we determined a racemization rate constant of 3.4×10^{-4} min^{-1} for the protiated peptide, a value about 1.14-fold higher than that previously measured (Geiger and Clarke 1987). When the data for the deuterated asparagine peptide was similarly analyzed, we found a racemization rate constant of 7.4×10^{-5} min^{-1}, which is almost five-fold lower than that found for the protiated peptide.

The rate at which racemized residues accumulated in both the protiated and deuterated peptides is seen in Fig. 4 to be biphasic; the accumulation is rapid over the first four days, and then slower between six and twenty days. This occurs because the asparaginyl residue in the protiated peptide VYPNGA forms a succinimide about 37-times more rapidly than does the aspartyl residue in VYPDGA (Stephenson and Clarke 1989). As shown in Fig. 2, the protiated and deuterated asparaginyl residues are 80% deamidated after three days and about 95% deamidated after 6 days, and thus the slower accumulation of racemized residues after six days reflects the slower succinimide formation of the now predominant aspartyl and isoaspartyl residues. Within the first day of aging under physiological conditions, well before half of the asparaginyl residues have deamidated, the rate of racemization of the protiated peptide is 3.65-times faster than that of the deuterated peptide. By 4 days of aging, when only about 14% of the peptides remain undeamidated, 5% of the Asn/Asp/isoAsp residues in the protiated peptide are racemized to the D-configuration, while only 1% of the deuterated Asn/Asp/isoAsp residues are racemized.

It is also of interest that when the deuteron comes off the alpha carbon, there is a 50% chance that the proton that replaces it will come back to the same side of the alpha carbon, regenerating the L-succinimide, but now with only the two deuteriums on the methylene group remaining. Thus, there will now be a mixture of L-succinimide with three deuteriums and with two deuteriums, and these can hydrolyze to form L-isoaspartyl and L-aspartyl residues with three and two deuteriums, respectively. The deuterium is slowing down racemization because it is less likely to come off the alpha carbon than is a hydrogen. However, once the deuteron has come off and is replaced by a proton from the water, the rate of racemization will increase to the rate of racemization previously reported by Geiger and Clarke (1987). This L-succinimide with an alpha hydrogen but with a deuterated methylene group will presumably racemize as quickly as will a fully protonated L-succinimide. However, because the aspartyl/isoaspartyl/succinimidyl residues in the deuterated peptide

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are only 1.49% racemized to the D-configuration after 21 days of incubation, only $2 \times 1.49\% = 2.98\%$ of the succinimides generated during this incubation have lost the deuterium from the alpha-carbon. The fact that over 97% of this deamidated peptide is still deuterated on the alpha-carbon is observed in Fig. 4 by the slower rate of racemization between 9 and 22 days of incubation. The slope of this line for the deamidated deuterated peptides is about 1.70×10^{-4} day^{-1}, and the slope for the deamidated protonated peptides is 6.77×10^{-4} day^{-4}. Thus, the rate of racemization of L-aspartyl/L-isoaspartyl residues is about 4-fold slower when these residues arise from deuterated L-asparaginyl residues.

Conclusions

We show here that the replacement of hydrogen atoms with deuterium atoms can markedly reduce the degree of spontaneous racemization in a model asparagine-containing peptide. Although such replacement does not decrease the rate of deamidation and has little effect on the consequent rate of formation of isomerized residues, isomerization damage can be readily reversed in most cells by the action of the protein repair methyltransferase, which converts L-isoaspartyl residues to normal L-aspartyl residues. The repair of D-aspartyl residues, however, is much less efficient, and leads to the generation of D-isoaspartyl residues (Lowenson and Clarke 1992, Young et al. 2005) so that preventing D-aspartyl formation in the first place by deuteration may be important in stabilizing protein and peptide pharmaceuticals and cellular proteins. The finding reported here suggests a potential intervention strategy to minimize this damage.

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Abbreviations

Fmoc	Fluorenylmethyloxycarbonyl
KIE	Kinetic isotope effects
Trt	Triphenylmethyl

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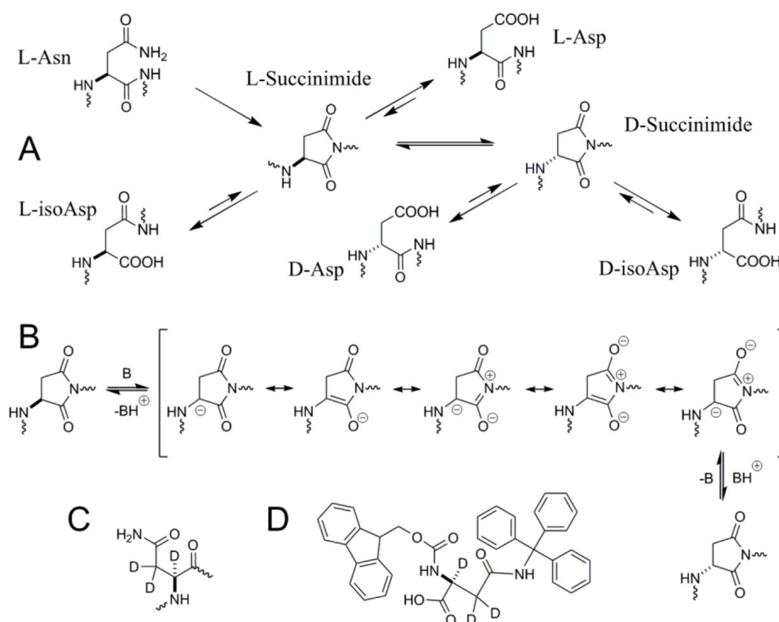

Fig. 1.

a Major pathway for the spontaneous degradation of L-asparaginyl residues in proteins. **b** Resonance forms of the succinimide product of asparagine deamidation showing the stabilization of the carbanion formed after removal of the proton on the alpha-carbon atom (modified from Radkiewicz et al. 1996). **c** Structure of the deuterated asparaginyl residue incorporated into the VYPNGA peptide. **d** Structure of d3-Fmoc-L-Asn(Trt)-OH, the protected deuterated asparagine substrate for peptide synthesis.

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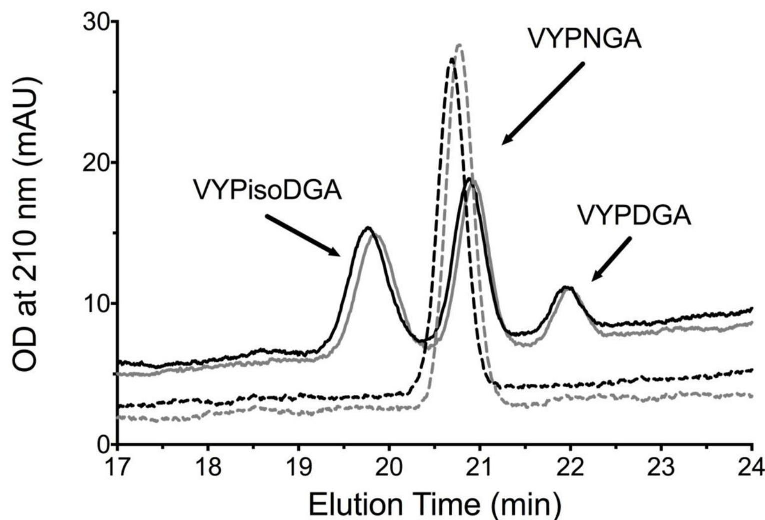

Fig. 2.

Deamidation of deuterated asparagine in a synthetic peptide results in the generation of isoaspartyl- and normal aspartyl-containing peptides. Unaged VYPNGA (1.3 nmol) containing protiated (dashed black line) and deuterated (dashed gray line) asparagine residues are found to coelute on a reverse-phase C18 HPLC column (Alltech Econosphere, 5 μm beads, 250 × 4.6 mm) using a Hewlett Packard 1090 series II Liquid Chromatography system and monitoring UV absorption at 210 nm. Following incubation at 37 °C and pH 7.4 for 38.8 h, the amount of protiated (solid black line) and deuterated (solid gray line) asparagine-containing peptide (1.3 nmol) decreases as they are converted to aspartyl- and isoaspartyl-containing peptides. The L- and D- forms of these peptides are not separated under these conditions. These HPLC runs were done with a linear gradient from 100% buffer A (0.1% trifluoroacetic acid in water) to 86% buffer A/14% buffer B (0.1% trifluoroacetic acid, 0.9% water, 99% acetonitrile) in 28 min.

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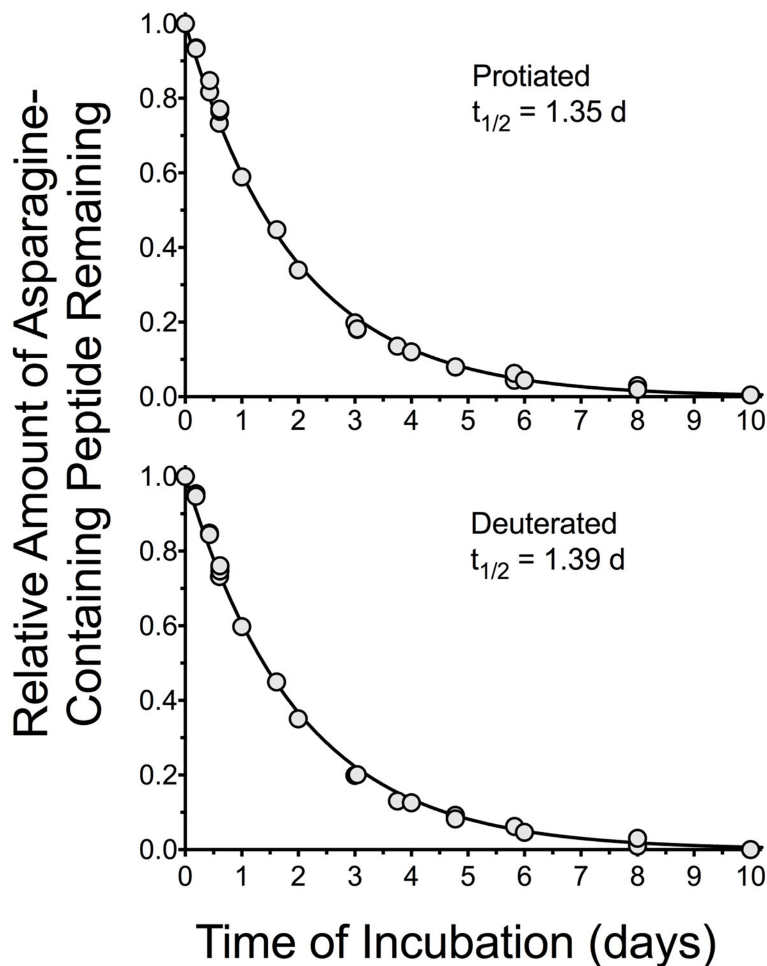

Fig. 3.

Deuteriums on the side chain methylene carbon and the alpha carbon of an asparaginyl residue have little effect on its rate of deamidation in a synthetic peptide. The peptide VYPNGA with either a protiated (upper panel) or deuterated (lower panel) asparaginyl residue was incubated at 37 °C in 0.1 M sodium phosphate pH 7.4 for various times. The relative quantity of asparagine-containing peptide remaining (open circles) was quantified by UV absorbance at 210 nm during separation of the peptides by HPLC using a reverse phase C18 column as shown in Fig. 2. There are 24 data points in each panel though some are concealed due to overlap, combined from two independent aging experiments. The line in each panel was fit to the data points using the first order reaction equation $[A] = [A]_0e^{-kt}$ and least squares analysis. The half-lives of the peptides were calculated from the best-fit lines.

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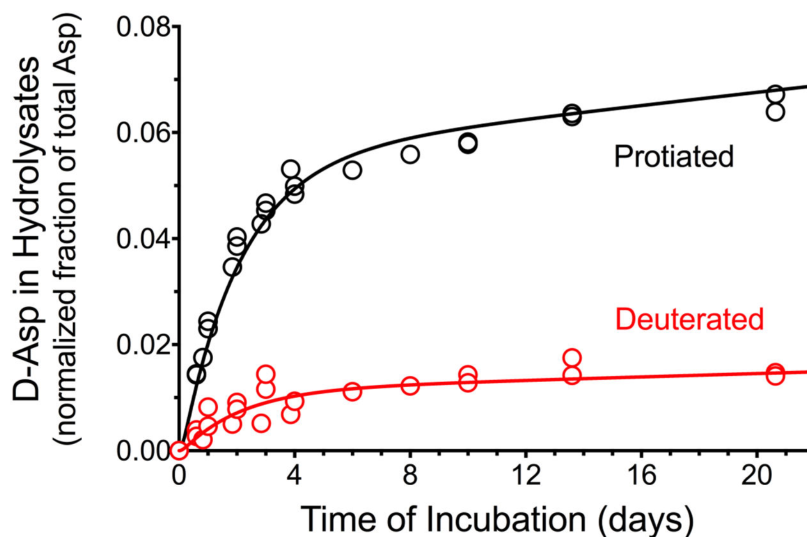

Fig. 4.

The peptide containing deuterated asparagine accumulates racemized aspartate/isoaspartate/succinimide at a much slower rate than does the protiated peptide. VYPNGA containing either protiated or deuterated asparagine was incubated for various times at 37 °C and pH 7.4 and then acid hydrolyzed. The amount of D-aspartate and L-aspartate in the hydrolysates was quantified following derivatization with ortho-phthalaldehyde and N-acetyl-L-cysteine and separation of the resulting fluorescent diastereomers by reverse-phase HPLC, and graphed as the fraction of D-aspartate relative to the total aspartate (open circles). Each data set contains 24 measurements combined from two independent aging experiments; some of these data points are not visible due to overlap. The lines were obtained by computer simulation of the reactions involved in deamidation of asparaginyl residues starting with previously-published kinetic constants (Geiger and Clarke 1987), and varying the racemization rate of the succinimide intermediate to give a best fit line as determined by least squares analysis.