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Asparagine Deamidation and the Role of Higher Order Protein Structure

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The 'protein world' exhibits additional complexity caused by post-translational modifications. One such process is nonenzymic deamidation of asparagine which is controlled partly by primary sequence, but also higher order protein structure. We have studied the deamidation of an N-terminal peptide in muscle glyceraldehyde 3-phosphate dehydrogenase to relate three-dimensional structure, proteolysis, and deamidation. This work has significant consequences for identification of proteins using peptide mass fingerprinting.

Keywords: Deamidation • proteolysis • protein structure • asparagine • aspartic acid • peptide mass fingerprinting

Introduction

The emergence of new analytical methods for protein characterization has led to the recognition that there is an additional dimension of complexity in the protein world created by a wide range of post-translational modifications. Some of these modifications are specific and are part of the obligatory maturation process of a protein, such as the removal of propeptides. Other changes are transient, reversible, and may only operate on a subset of molecules in the protein pool (the best understood is phosphorylation). Other irreversible changes, such as deamidation or lysine aldehyde mediated cross-linking, are nonenzymic, and the longevity of the protein may be reflected in the accumulation of such changes.

Deamidation of the side chain of asparagine residues is a nonenzymic process[1] (www.deamidation.org). The conversion of asparagine to aspartic acid or isoaspartic acid elicits a local change in charge, and has the potential to impose a self-timer on protein molecules, altering activity or stability with lifetime kinetics.[2–5] The ability to include a nonenzymic irreversible change into a protein that elicits a small steric change but a substantial local alteration in electrostatic potential could provide an opportunity to evolve a programmable irreversible change of state into a protein. Most studies on asparagine deamidation have been conducted with model peptides[6] which are essentially devoid of higher order structure and which permit the peptide backbone and side chain to adopt a conformation compatible with the cyclic intermediate that is required for this reaction to take place. Since the flexibility and conformational freedom of the peptide is modified by the nature of the amino acids, the rate of deamidation of model peptides is strongly influenced by the flanking residues[6] and the primary influence on the rate of asparagine deamidation is the amino acid C-terminal to the asparagine residue. From studies of model peptides, the highest rate of deamidation is obtained when the carboxyl neighbor is glycine, yielding a half-time for deamidation of around 24 h.[6] This is probably because the lack of a Cβ atom minimizes steric hindrance and permits ready formation of the five-membered imide conducive to the deamidation reaction. The N-terminal neighbor has a minor effect on the rate of deamidation.[6] While most of our understanding of rates of peptide deamidation has derived from short, model peptides, the same sequences, when incorporated into protein structures, might acquire a relatively immobile backbone trajectory that could constrain the sequence to either favor or disfavor deamidation.

The resolution of modern mass spectrometers used routinely in proteomic analyses permits ready resolution of the monoisotopic peptide–ion from the ^{13}C isotopomer variants, even at charge states of +2 or +3. At this level of resolution, a deamidation event (Asn → Asp) would be readily recognized, as it elicits a mass shift of +0.985 Da (-NH_2 = 16.03 to -OH = 17.01). In circumstances where a peptide exists as a mixture of the amide and cognate acid species, a complex mass spectrum would ensue that appears as an atypical isotopomer distribution for a peptide of that mass. It follows that partial deamidation events should be readily observed by examination of the atypical profile, particularly without prior chromatographic separation that would resolve the amide and cognate acid in chromatographic space.

In the course of proteomics studies of soluble proteins in skeletal muscle,[7] we observed that a peptide from one protein in particular exhibited a noticeable and atypical natural isotope distribution profile, consistent with a mixture of an asparagine-containing peptide and the cognate deamidation product. This peptide was derived from the N-terminus of an abundant protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We present here a comprehensive analysis that confirms that the 'atypical' isotope profile is in fact attributable to partial deamidation of an asparagine residue. Deamidation of -AsnGly-

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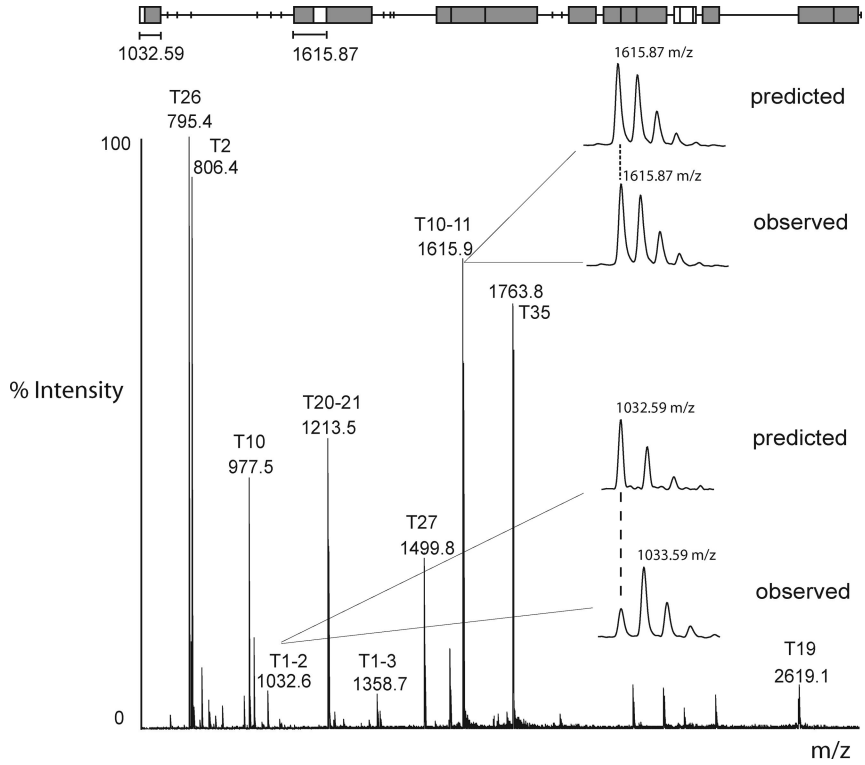

Figure 1. Atypical peptide mass spectrum consistent with deamidation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1 mg/mL diluted to 0.2 mg/mL with 50 mM ammonium bicarbonate) purified from rabbit skeletal muscle (Sigma, Dorset, U.K.) was digested in solution with trypsin at a substrate/protease ratio of 100:1 by weight, and the masses of the resultant tryptic peptides were assessed by MALDI-ToF mass spectrometry; a coverage map is included at the top of the figure, with identified peptides indicated by a shaded block and those identified as part of a missed cleavage by an open block. The spectrum of a typical partial cleavage tryptic peptide (T10–11, *m/z* 1615.9) was compared with the mass spectrum predicted by the MS-Isotope tool (http://prospector.ucsf.edu/). This behavior, common to almost all other peptides, emphasized the atypical profile observed for the N-terminal partial cleavage peptide (T1–2, *m/z* 1032.6).

sequences occurs during sample preparation in proteomics,[8] and proteolysis conducted at lower pH and temperature will minimize artifactual deamidation.[9] Here, we show that deamidation is constrained by higher order structure and is enhanced after release of that conformational restraint by proteolysis. This observation has significance for the identification of deamidation events by protein or peptide mass spectrometry[10–12] and reinforces the role that protein conformation can play in this process.

Experimental Section

Materials and Reagents. Trypsin (sequence grade) was obtained from Roche Diagnostics (Lewes, U.K.). All other chemicals and solvents (HPLC grade) were purchased from Sigma-Aldrich Company Ltd. (Dorset, U.K.) and VWR International Laboratory Supplies (Leicestershire, U.K.).

One-Dimensional Gel Electrophoresis (1DGE). Purified GAPDH from rabbit skeletal muscle (Sigma, Dorset, U.K.) (10 μg) was electrophoresed through a 12.5% polyacrylamide gel and visualized with Biosafe Coomassie Brilliant Blue stain (Bio-Rad, Hemel Hempstead, U.K.). Gels were destained with a 10% acetic acid 10% methanol solution.

In-Gel Trypsin Digestion. Gel plugs containing GAPDH (identification confirmed by MALDI-ToF MS, results not shown) were excised from 1D gels using a glass pipet and transferred to an Eppendorf tube. To each tube, 25 μL of 50 mM ammonium bicarbonate, pH 8.2, and 50% (v/v) acetonitrile (ACN) was added and incubated at 37 °C for 20 min. This process was repeated until all of the stain had been removed. The plugs were then washed in 50 mM ammonium bicarbonate, which was subsequently discarded. The gel was dehydrated using 5 μL of ACN, and incubation at 37 °C was resumed for 30 min. Once dry, the gel was rehydrated in 50 mM ammonium bicarbonate (9 μL) containing trypsin (1μL of 100 ng/μL trypsin stock reconstituted in 50 mM acetic acid), and digestion was allowed to continue overnight at 37 °C; the digestion was halted by the addition of 2 μL of formic acid.

MALDI-ToF Mass Spectrometry. Peptides were analyzed by MALDI-ToF (M@LDI; Waters, Manchester, U.K.) mass spectrometry. For this, 1 μL of digested material was mixed with an equal volume of α-cyano-hydroxycinnamic acid in 50% (v/v) ACN and 0.1% (v/v) trifluoroacetic acid. This was allowed to dry, and peptides were acquired over the range 900–3000 *m/z*. For each combined spectrum, 20–30 spectra were acquired (laser energy typically 30%) with 10 shots per spectrum and a laser firing rate of 5 Hz. Data were processed using MassLynx software to subtract background noise using polynomial order 10 with 40% of the data points below this polynomial and a tolerance of 0.01. Spectral data were also smoothed by performing two mean smooth operations with a window of three channels. To confirm the assumption that both acid and amide forms of the peptide ionize with equal signal response in MALDI-ToF MS, the synthetic peptide for the amide form was allowed to fully deamidate (by incubation at 37 °C) and mixed in a known ratio with asparagine-containing peptide in a strong acidic solution to prevent further deamidation. The signal response from the two variants was identical.

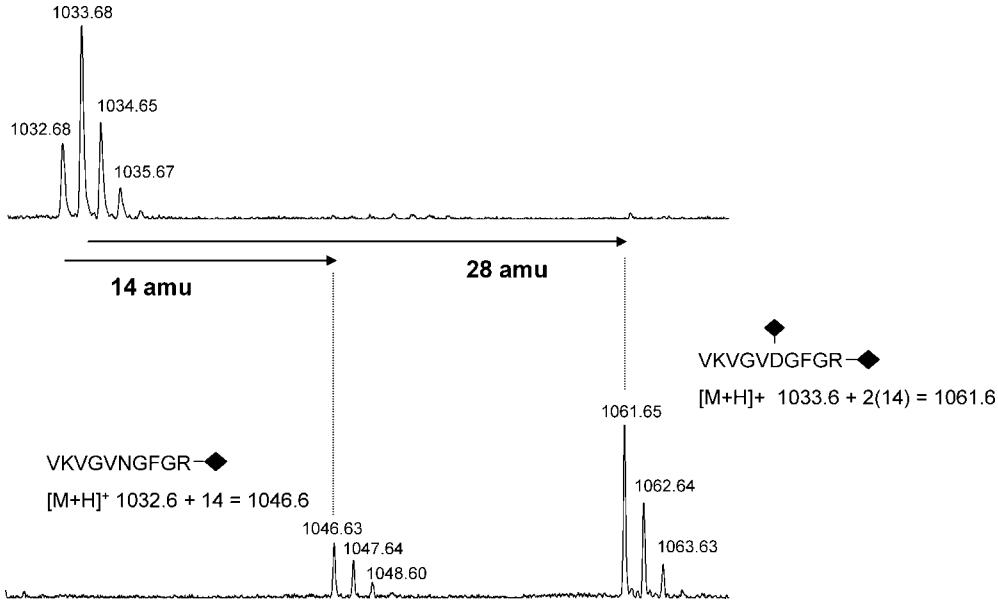

Figure 2. Esterification of acidic residues in the N-terminal peptide of GAPDH. Tryptic peptides recovered from an in-gel tryptic digest of GAPDH (purified from rabbit skeletal muscle, Sigma, Dorset, U.K.) were reacted with acetyl chloride and methanol to convert acidic residues to their corresponding methyl esters. The upper mass spectrum is the peptide resulting from partial deamidation of Asn_6, thus, is a mixture of two forms (asparagine containing and aspartic acid containing). The lower spectrum, obtained after esterification has resolved the peptide into two distinct reaction products at 1046.63 *m/z* and 1061.65 *m/z*, consistent with the addition of one and two methyl groups (+14.03 Da), respectively.

In-Solution Tryptic Digestion. Soluble protein (purified GAPDH; 1 mg/mL) was diluted 10-fold with 50 mM ammonium bicarbonate prior to addition of trypsin (100:1 substrate/protease). The reaction mixture was incubated at 37 °C for 24 h, and peptides were analyzed by MALDI-ToF MS.

Esterification of Peptides. A stock solution of methanol (1 mL, previously stored at −20 °C for 15 min) and acetyl chloride (150 μL) was prepared. An aliquot (10 μL) of this mixture was then added to a dried portion of the peptide pool recovered after in-gel digestion of the protein. The mixture was incubated at room temperature for 45 min prior to drying in a vacuum centrifuge. Esterified peptides were analyzed by MALDI-ToF MS.

Monitored Proteolysis of GAPDH. Digestion reaction mixtures with trypsin were stopped at selected time points after addition of enzyme by removing 10 μL and adding to an equal volume of 10% (v/v) formic acid. The fractions were subsequently stored at −20 °C until the end of the time course. Peptides were analyzed by MALDI-ToF MS.

Data Processing. The natural isotope profile for the acid VKVGVDGFGR and amide VKVGVNGFGR variants of the GAPDH N-terminal peptide were predicted using the MSIsotope tool provided online within the Protein Prospector Package (http://prospector.ucsf.edu/ucsfhtml4.0/msiso.htm). The intensities of each isotopomer peak were added, and the combined theoretical spectrum was compared with the intensities derived from the experimental mass spectrum. The sum of the squares of the deviation between predicted and experimental data was used to generate the object function, and the sole parameter (P_A) was the proportion of the acidic component (by definition, equal to $1 - P_N$, where P_N is the proportion of amide). The nonlinear optimization function (Solver) within Excel was used to obtain the best fit value of P_A. Additionally, some samples were analyzed by a high speed spectrum deconvolution tool, implemented as computer hardware in a field programmable gate array.[13]

Absolute Quantification of Proteolysis Using a Stable Isotope-Labeled Synthetic Peptide. The N-terminal peptide of GAPDH, of sequence VKVGVNGFGR and neutral mass 1041.59 Da, was synthesized by Sigma-Genosys (Dorset, U.K.) and was labeled at the arginine residue with both [$^{13}C_6$] and [$^{15}N_4$] giving a 10 Da mass offset from the analyte peptide. For quantification of proteolysis, the synthetic peptide was added to digested material in 10% (v/v) formic acid to stop digestion and deamidation. Peptides were analyzed by MALDI-ToF MS, and the relative intensities of analyte peptide and internal standard were used to quantify the amount of peptide released from the protein during incubation with trypsin at 37 °C. As conversion of asparagine to aspartic acid alters the isotope envelope of the analyte peptide, the composite abundance of the entire isotopic envelope for both analyte and internal standard peptide was summed in each case. These data permitted the kinetics of proteolytic release of the N-terminal peptide from GAPDH to be calculated and were used along with the kinetics of deamidation to investigate the interaction between these two alternative processes. The rate of deamidation was measured across the time course of digestion by calculating the proportion of acid and amide variants of the peptide at each time point. This was done during proteolysis of GAPDH and for the synthetic peptide, at different temperatures.

Results and Discussion

One of the most abundant soluble sarcoplasmic proteins in skeletal muscle is glyceraldehyde 3-phosphate dehydrogenase, amounting to 11 ± 1% (mean ± SEM, $n = 3$) of soluble protein when resolved by 1D gel electrophoresis (1DGE) and analyzed by densitometry (data not shown) and up to 500 ± 50 nmol/g (mean ± SEM, $n = 4$) tissue when analyzed using the QconCAT method for absolute quantification.[13–15] MALDI-ToF spectra

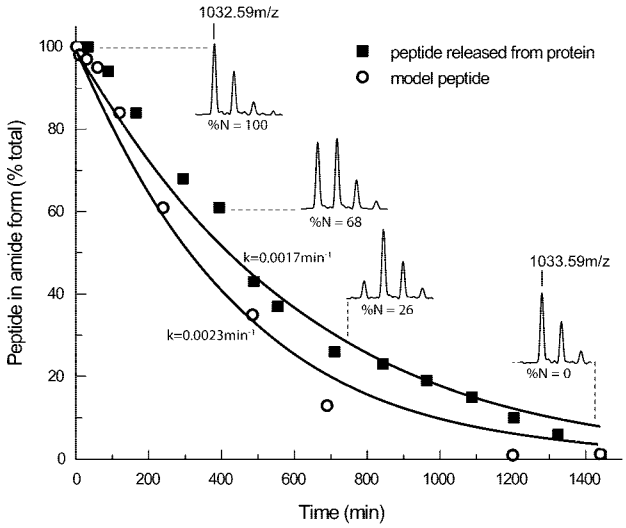

Figure 3. Time course of deamidation of the N-terminal peptide of GAPDH. Purified rabbit skeletal muscle GAPDH (Sigma, Dorset, U.K.; 1 mg/mL diluted to 0.2 mg/mL with 50 mM ammonium bicarbonate) was digested with trypsin (trypsin/protein 1:100) over 24 h at 37 °C. Proteolysis was stopped at 0, 2, 5, 10, 30, 60, 120, 240, 480, and 1440 min by mixing 10 μL from the digestion mixture with 10 μL of 10% (v/v) formic acid. The resulting peptides were analysed by MALDI-ToF mass spectrometry, and deamidation was monitored during proteolysis for the N-terminal peptide of sequence VKVGVNGFGR at 1032.59 *m/z*. The proportion of acid and amide variants was assessed as described in Experimental Section, from peak height data, and plotted as a function of time (closed squares). Peptide envelopes illustrating the conversion of acid to amide form in MALDI-ToF mass spectra corresponding to time points over 24 h are inserted above the data. To compare this with model peptide studies, the N-terminal peptide of GAPDH, of sequence VKVGVNGFGR and mass 1041.59 Da, was synthesised by Sigma-Genosys (Dorset, U.K.) and was labelled at the arginine residue with both [$^{13}C_6$] and [$^{15}N_4$] giving a 10 Da mass offset from the analyte peptide. This peptide was incubated in 50 mM ammonium bicarbonate at 37 °C, and a sample of the peptide was added to an equal volume of 10% (v/v) formic acid at selected time points. The relative amounts of acid and amide variants of the peptide were measured using MALDI-ToF MS, and this was used to calculate the rate of deamidation. These data are presented as open circles. The solid lines are the trajectories taken by first-order decay for the synthetic peptide and the proteolyzed glyceraldehyde 3-phosphate dehydrogenase.

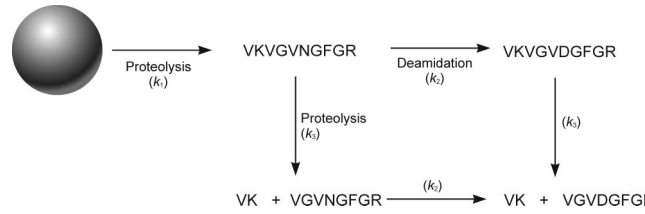

Figure 4. Model of proteolysis and deamidation of the N-terminal peptide of GAPDH. The simultaneous processes of proteolysis and release of the N-terminal peptide of GAPDH followed by deamidation of the asparagine residue to aspartic acid were modelled according to this scheme. The model also included the subsequent proteolysis of the N-terminal peptide (**VKVGVNGFGR** or **VKVGVDGFGR**) at the internal arginine residue to generate a dipeptide and a truncated peptide (**VK**+**VGVNGFGR** or **VK**+**VGVDGFGR**). In this scheme, we assumed that the rate of deamidation was the same, whether in the full length or truncated N-terminal peptide, and that the rate of removal of the N-terminal dipeptide was independent of the amide/acid variants.

for this protein, isolated by 1DGE and digested with trypsin prior to MS analysis are of high quality, give very high probability identification of this protein (not shown), and yield approximately 20 peptides, ranging from 805.5 *m/z* to 2265.4 *m/z*. Close inspection of each peptide indicated that for most, the observed mass isotopomer distribution was as expected, and was in close agreement to the distribution predicted by the MsIsotope program (http://prospector.ucsf.edu/). One peptide in particular (VKVGVNGFGR, $[M+H]^+$ 1032.58 *m/z*) was notably different from the others, inasmuch as the isotope distribution profile was far removed from the predicted profile (Figure 1). In particular, the relative intensity of the monoisotopic ion was diminished, and of lower intensity than the first [^{13}C] isotopomer, a relative intensity pattern that is unexpected for a peptide of mass 1031.58 Da, given an empirical formula of $C_{46}H_{78}N_{15}O_{12}$.

The mass isotopomer envelope is consistent with the analyte being a mixture of two peptides, one of monoisotopic *m/z* 1032.58 and a second at a monoisotopic *m/z* of 1033.58. The higher *m/z* peptide could have been a contaminant or it could have been generated from the peptide at *m/z* 1032.58. In the latter case, the most probable explanation for the mass increase was deamidation of the asparagine residue, which, by conversion to an aspartate residue, would increase the mass by 0.985 Da (-NH_2 to -OH). To prove that the atypical profile was a consequence of deamidation, we esterified the peptide mixture to convert carboxyl groups to their methyl esters. The mass shift on esterification would be 14.03 Da. Because the peptide **V_2KVGVNGFGR$_{10}$** would possess a single carboxyl group in the amide form (the alpha carboxyl group), and two in the acid form, esterification should therefore deconvolute the atypical peptide into two products, one esterified at a single position (+14.03 Da), and a second modified in two positions (+28.06 Da). When the peptide mixture was analyzed after esterification, the MALDI-ToF ions in the 1032−1036 *m/z* region disappeared, and two new ions appeared, one representing the single modified amide (*m/z* 1032.58 + 14.03 = 1046.61) and the second reflecting the double modified acid (*m/z* 1033.58 + 28.06 = 1061.64; Figure 2).

From this analysis, it was not possible to assess whether the residue had deamidated *in vivo* or was an artifact of sample preparation and processing. To assess the extent of deamidation of this peptide in the native protein, we treated purified rabbit GAPDH with trypsin and monitored the proteolysis and the partition between the acid and amide variants of the peptide in MALDI-ToF mass spectra (Figure 3; the same experiments were repeated for an in-solution tryptic digest of chicken skeletal muscle soluble proteins and the same behavior was apparent, results not shown). The N-terminal peptide of GAPDH (**VKVGVNGFGR**) was released within a few minutes and was readily detected as the first analyte ion to appear in the MALDI-ToF spectrum. In the early stages of digestion, the mass spectrum of this peptide was entirely consistent with it being exclusively in the amide form. However, as time progressed during proteolysis, the mass spectrum of the peptide showed that the peptide was converted to a mixture of the amide and acid variants, and after 10 h of digestion, the peptide was over 80% in the acid form. The first-order rate constant for this process was approximately 0.0017 min^{-1}, which was higher than the value derived from model peptides; for the sequence NH_2**GVNGG**OH, the first-order rate constant was

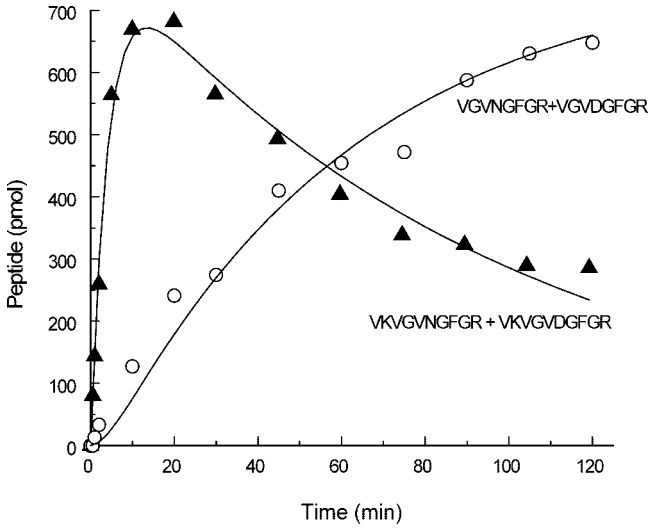

Figure 5. Absolute quantification of proteolysis of the GAPDH N-terminus. Purified rabbit skeletal muscle GAPDH (Sigma, Dorset, U.K.; 1 mg/mL diluted to 0.2 mg/mL with 50 mM ammonium bicarbonate) was digested with trypsin (trypsin/protein 1:10) over 24 h at 37 °C. The N-terminal peptide of GAPDH, of sequence **VKVGVNGFGR** and mass 1041.59 Da, was synthesised by Sigma-Genosys (Dorset, U.K.) and was labelled at the arginine residue with both [$^{13}C_6$] and [$^{15}N_4$] giving a 10 Da mass offset from the analyte peptide. For quantification of proteolysis, the synthetic peptide was added to digested material in 10% (v/v) formic acid to stop digestion at selected time points. Peptides were analyzed by MALDI-ToF MS, and the relative intensities of analyte peptide and internal standard were used to quantify the amount of peptide released from the protein during incubation with trypsin at 37 °C. Both the N-terminal peptide (**VKVGVNGFGR/VKVGVDGFGR**; *m/z* 1032.59 $[M+H]^+$; closed triangles) and the shorter peptide produced by further proteolysis (**VGVNGFGR/VGVDGFGR**; *m/z* 805.59 $[M+H]^+$; open circles) were monitored. As conversion of asparagine to aspartic acid alters the isotope envelope of the analyte peptide, the composite abundance of the entire isotopic envelope for both analyte and internal standard peptide was summed in each case. The solid lines reflect the fitted curves for the transient appearance of the N-terminal peptide (**VKVGVNGFGR/VKVGVDGFGR**) and the truncated product (**VGVNGFGR/VGVDGFGR**), modelled and fitted as sequential first-order reactions (see text).

previously measured at 0.0004 min^{-1}.[16] However, the buffer conditions for the two experiments are not identical, and pH has a large affect on deamidation rate. The rate of deamidation under these buffer conditions was confirmed using a synthetic peptide of the same sequence; for this peptide, the rate of deamidation was 0.0023 min^{-1}. The higher rate of deamidation of the synthetic peptide might reflect an association between the partially digested protein and the N-terminal peptide which introduced a degree of conformational 'freezing' of the peptide, diminishing the deamidation rate, but this remains conjecture at present.

To investigate the kinetics of both deamidation and proteolysis, a synthetic peptide of sequence VKVGVNGFGR, mass 1041.59 Da, was synthesized and was labeled at the arginine residue with both [$^{13}C_6$] and [$^{15}N_4$] giving a 10 Da mass offset relative to the natural peptide. This peptide, identical to the N-terminal peptide of GAPDH, was used to monitor the behavior of the peptide, and for quantification.[17] Because the N-terminal peptide itself contains an internal tryptic cleavage site (**VK - VGVDGFGR**), the peptide **VKVGVDGFGR** (summed across acid or amide forms) decreased slowly as digestion continued. We created a model (Figure 4) that took into account the sequential first-order processes of proteolysis (k_1) of the native protein (N_{native}) to release the amide form of the peptide (**VKVGVNGFGR**) followed by deamidation (k_2) to generate the acid form (**VKVGVDGFGR**).

$$N_{native} \xrightarrow{k_1} \text{VKVGVNGFGR} \xrightarrow{k_2} \text{VKVGVDGFGR}$$

Furthermore, the model also included a secondary process of proteolysis of the released peptide in either the acid or amide form to release the ValLys dipeptide. The rate of appearance of the deamidated peptide is given by

$$\text{VKVGV(N/D)GFGR} \xrightarrow{k_3} \text{VK} + \text{VGV(N/D)GFGR}$$

We assumed that the rate of deamidation (k_2) was independent of the N-terminal ValLys dipeptide and that the rate of tryptic removal of the N-terminal dipeptide (k_1) was the same, irrespective of whether the peptide was in acid or amide form. The change in amount (relative to the initial amount of protein, $N_{native(t=0)}$) of the larger peptides (**VKVGVNGFGR** + **VKVGVDGFGR**, $N + D$) as a function of time, is given by

$$N + D = N_{native}\left(\frac{k_1}{k_3 - k_1}(e^{-k_1 t} + e^{-k_3 t})\right) \quad (1)$$

As part of the same process, the shortened peptide (**VGVNGFGR** + **VGVDGFGR**, $N' + D'$) appears according to

$$N' + D' = N_{native}\left(1 - \frac{k_3}{k_3 - k_1}e^{-k_1 t} + \frac{k_3}{k_3 - k_1}e^{-k_3 t}\right) \quad (2)$$

Assuming that the rate of tryptic cleavage is consistent for both acid and amide variants, from these equations, we were able to calculate the second-order rate constants (first-order rate constant divided by protease concentration) for initial release of the large peptide (k_1) and the rate of proteolysis of this large peptide (k_3) (Figure 5). The value of k_1 was estimated to be 1.22 ± 0.025 $min^{-1} \cdot \mu M$ and for k_3, 0.50 ± 0.008 $min^{-1} \cdot \mu M$ (trypsin = 0.2 μM). As expected, the endoproteolytic release of the longer peptide is faster than the release of the N-terminal dipeptide, as trypsin is known to act poorly as a dipeptidyl peptidase. However, the release of the longer peptide is likely to be suppressed by the three-dimensional structure of the protein.

To investigate the effects of the higher order structure of GAPDH on proteolysis and subsequent deamidation, we analyzed the X-ray crystal structure of rabbit GAPDH (PDB code 1J0X.PDB). First, we used the tool NickPred,[18] which although designed to predict sites of proteolytic attack, can generate a comprehensive analysis of the environment of every residue in a protein sequence. The N-terminal region of GAPDH is rather constrained, exhibiting low temperature factors (*B*-values) and low protrusion and accessibility (results not shown). Close inspection of the structure in the vicinity of Asn_6 revealed this region of the polypeptide chain was folded in an extended β configuration, constrained by 14 hydrogen bonds in a network that might be expected to constrain main chain flexibility and therefore reduce the propensity for asparagine deamidation (Figure 6). However, once the peptide was released by proteolysis, deamidation proceeded at a higher rate than that predicted from model studies. These experiments are consistent with the following propositions; that the residue in

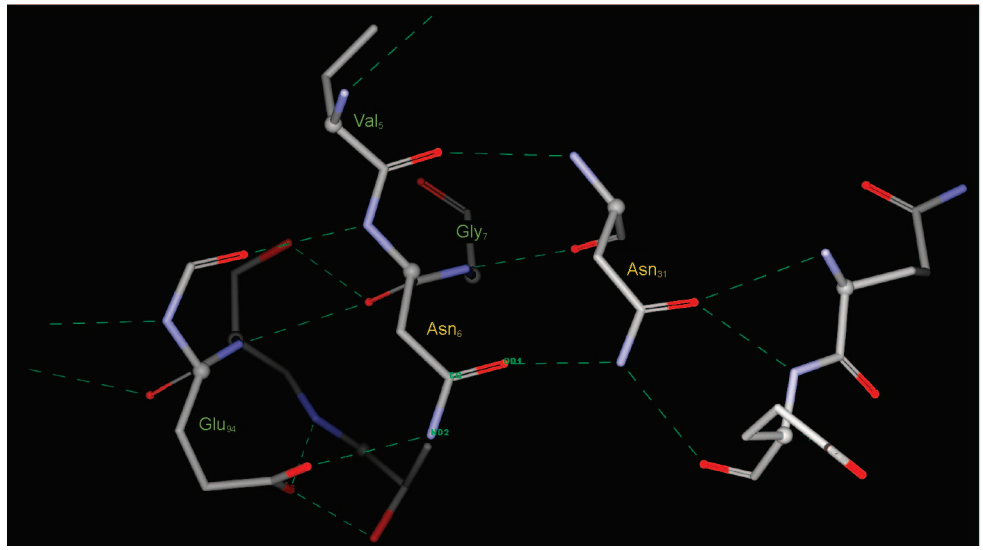

Figure 6. 3D structure of rabbit skeletal muscle GAPDH. X-ray crystal structure of the N-terminal region of rabbit skeletal muscle GAPDH (PDB code 1J0X) highlighting the Asn_6Gly_7 deamidation site and the local hydrogen bonded environment. The green dashed lines denote hydrogen bonds.

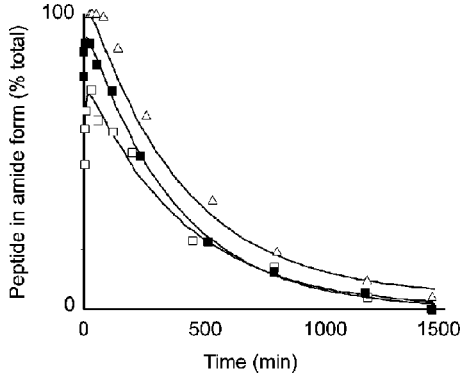

Figure 7. The effect of denaturing protein structure by heating on the rate of deamidation. GAPDH (1 mg/mL diluted to 0.2 mg/mL with 50 mM ammonium bicarbonate) purified from rabbit skeletal muscle (Sigma, Dorset, U.K.) was digested with trypsin in solution at a ratio trypsin/protein 1:100 at 37 °C for 24 h. Prior to digestion, GAPDH was incubated for 1 h at 4 °C (open triangles), 1 h at 60 °C (closed squares), and 1 h at 60 °C followed by 24 h at 37 °C (open squares). For each, deamidation was monitored over 24 h proteolysis and the proportion of acid and amide was calculated from the relative peak intensities of the two ions in MALDI-ToF mass spectra.

the intact protein is exclusively in the amide form, that the tryptic fragment containing the amide residue can undergo deamidation, and that deamidation is not an artifact of the mass spectrometric analysis. Excision of the peptide from the GAPDH structure relieves the constraint in the peptide backbone trajectory, permitting the deamidation reaction to take place. It followed therefore that prior denaturation of the protein might permit deamidation prior to digestion with trypsin. We conducted experiments in which we denatured GAPDH by heating to 60 °C for 1 h before proteolysis (Figure 7), a denaturation treatment that was not sufficient to cause the protein to precipitate. Subsequently, when trypsin was added, the N-terminal peptide was again released rapidly, and the proportion of amide and acid variants of the peptide was assessed as previously described. Under these circumstances, the peptide first released was approximately 80% amide, with a significant proportion of acid form being measurable. This contrasted markedly with proteolysis of the native protein, when the peptide is initially all in the amide form. We attribute this behavior to the increased conformational flexibility of the peptide in the heat-treated protein, such that the peptide could acquire a conformation that allowed deamidation. Further, this unfolded and flexible component might be expected to be hypersensitive to proteolysis and to be released first. As the digestion proceeded, additional peptide in the amide form was released, and the proportion of amide therefore increased transiently, until the deamidation reaction dominated the peptide profile. When the functions derived previously were used, we obtained a value for deamidation of 0.0023 min^{-1}, in close agreement with that observed previously. If the heat-treated peptide was allowed to incubate at 37 °C for 24 h after the 60 min denaturation period at 60 °C, and then proteolyzed with trypsin, the peptide first released was now only 50% in the amide form, consistent with extensive deamidation prior to proteolysis, consequential to denaturation. Again, as expected, proteolysis led to the slower release of peptide that was constrained and unable to deamidate, and there was a transient increase in the proportion of amide which again decayed at the same rate as observed previously ($k_2 = 0.0024$ min^{-1}). The behavior of the system was consistent with the GAPDH preparation being 76% in the amide form, and 26% in a denatured form that was then rapidly proteolyzed to generate the free acid form of the peptide. The effect of denaturation on the availability of the N-terminal peptide of GAPDH for deamidation is quite striking and defines the importance of monitoring the two processes of proteolysis and deamidation simultaneously, especially as this effect is only observed upon loss of higher order structure, and not upon increasing concentration of protease (results not shown).

Conclusions

Deamidation is recognized as a potential source of microheterogeneity in protein structure, and it may play a significant role as a biological 'timer' that is mediated nonenzymatically.[1–5] Although many studies have emphasized the deamidation of short, flexible peptides, protein deamidation can be limited by higher order structure and might only occur at the peptide level

following proteolytic release.[8] The ease with which some peptides deamidated could then lead to the erroneous interpretation of a deamidation event as occurring in the intact protein. Difficulties of measuring deamidation have been discussed,[19] and analyses often use electrospray ionization mass spectrometry[6] and reversed-phase chromatographic matrices[8] to resolve acid and amide variants of a peptide, precluding analysis of complex mixtures. There is also considerable scope for MALDI sample ionization, which, when coupled with a simple esterification reaction, can clearly identify and characterize such deamidation variants. We suggest that there may be merit in closer examination of the isotope distribution profile of peptide mass fingerprints, to search for anomalies such as that noticed here. In particular, it is advantageous to monitor deamidation and proteolysis simultaneously when characterizing post-translational behavior of known proteins and peptides. This will also unravel information about the higher order structure of a protein, the influence of which not only on proteolysis but also on subsequent modifications to newly accessible regions of the protein, is paramount.

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