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Degradation of recombinant porcine growth hormone in the presence of guanidine hydrochloride

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Abstract

This study was conducted to investigate the effect of guanidine hydrochloride (Gdn HCl) on the rate of chemical degradation of recombinant porcine growth hormone (pGH) at alkaline pH. The protein was incubated in 0.2 M Tris buffer, pH 9 at 37°C containing Gdn HCl at concentrations ranging from 0 to 6 M. The conformation of pGH under the different solution conditions was assessed by circular dichroism spectropolarimetry. Degradation was monitored by reversed phase HPLC (RP-HPLC), size exclusion chromatography (SEC) and SDS-PAGE. An associated form of pGH was populated in the presence of 3 and 4 M Gdn HCl, whereas the protein was completely unfolded in 5 and 6 M Gdn HCl. The apparent degradation rate constant determined by RP-HPLC increased by a factor of 18 in the presence of 3 M Gdn HCl and by a factor of five in the presence of 6 M Gdn HCl relative to that in buffer alone. RP-HPLC and SEC data suggested that the product distribution also differed between buffer and solutions containing Gdn HCl. While the increased rate of degradation for the denatured state was not unexpected, the dramatic increase at an intermediate Gdn HCl concentration was surprising and indicated the importance of considering chemical instability when proteins are exposed to denaturants.

Keywords: Protein stability; Chemical stability; Porcine growth hormone

1. Introduction

Few studies have specifically addressed the relationship between protein conformation and rates and mechanisms of chemical degradation. As proteins are subjected to potentially denaturing

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conditions at various stages of production, assessment of this relationship is important when optimizing their development. For example, strong denaturants and pH extremes frequently incorporated to solubilize proteins during isolation from bacterial cell lines, induce significant conformational changes which may affect the rate of chemical degradation. Similarly, lyophilization which is often undertaken to prepare stable formulations, may expose proteins to denaturing conditions which may impact on rates of chemical degradation.

A major hurdle to investigating the relationship between protein conformation and chemical stability is the difficulty associated with isolation and characterization of conformationally altered forms. Gross conformational changes generally predispose proteins to subsequent physical instability such as aggregation, precipitation and adsorption to surfaces (Manning et al., 1989). Consequently, much of the current evidence of conformational effects on chemical stability is based upon knowledge of the location of labile amino acids within protein structures. For example, analysis of labile Asn residues in proteins of known crystal structure has shown that such residues in loop structures are far more susceptible to deamidation than when they reside in regions of ordered structure (Chazin and Kossiakoff, 1995), with the differences attributed to steric effects on the rate determining formation of the cyclic succinimide intermediate.

Stevenson et al. (1993) reported that the rate of deamidation of Asn^{8} in 32-residue analogues of bovine growth hormone releasing factor was related to their helical content. NMR and CD studies indicated that substitution of Pro at position 15 significantly decreased helical content whereas substitution of Ala at the same position enhanced the helix forming ability of the peptide. These changes in helical content were associated with marked increases and decreases, respectively, in the rate of deamidation of Asn^{8} in aqueous methanolic solutions providing evidence of the importance of secondary structure on the rate of deamidation.

The decreased susceptibility of Asn to deamidation when present in helical structures has also been reported for insulin (Brange and Langkjaer, 1992). The inclusion of phenol (and derivatives) in insulin formulations resulted in a significant reduction in deamidation of Asn^{B3}, which was attributed to the formation of an additional helical segment in the N-terminal region of the B chain which contained the labile residue. The importance of conformation on the rate of deamidation has also been demonstrated for ribonuclease A where reduction of the disulphide bonds and carboxymethylation of the Cys residues resulted in a dramatic increase in the rate of deamidation (Wearne and Creighton, 1989).

Conformational effects on the stability of Met residues and disulphide bonds in proteins have also been reported. For many proteins, oxidation of Met under native conditions only occurs at those Met residues which are accessible to the solvent. This is the case for bovine growth hormone (bGH) and human growth hormone (hGH) in that the complete oxidation of all Met residues requires the use of denaturing conditions (Glaser and Li, 1974; Houghten et al., 1977). Buckwalter et al. (1992) also reported that reduction and carboxymethylation of pGH resulted in selective modification of the exposed 'small loop' disulphide (Cys^{181}–Cys^{189}), whereas the partially buried 'large loop' disulphide (Cys^{53}–Cys^{164}) was not affected.

In the present study, the degradation of pGH, a 22 kD protein consisting of four antiparallel α-helices with two disulphide bonds, was monitored by RP-HPLC and SEC in the presence of varying concentrations of Gdn HCl. The three dimensional structure of pGH determined by X-ray crystallography has been published previously (Abdel-Meguid et al., 1987). Guanidine HCl was chosen to induce conformational changes in pGH on the basis of its widespread use in the solubilization of inclusion bodies isolated from bacterial cell lines, its high stability upon storage (unlike urea which forms reactive cyanate ions upon storage at alkaline pH (Hagel et al., 1971)), and its ability to solubilize denatured forms of pGH.

2. Materials and methods

2.1. Chemicals and reagents

Recombinant DNA-derived methionyl-pGH was supplied by Bresagen Ltd. (Adelaide, South Australia) as lyophilized protein. LC-MS analysis of this material indicated the presence of two peaks in a ratio of 4:1 in the reversed phase chromatogram with masses of 21856.9 ($\pm$0.7) and 21581.1($\pm$1.1), respectively. The molecular weight of 21856.9 agreed well with the theoretical mass of 21858.03 for Met-pGH, and the species with a molecular weight of 21581.1 was tentatively identified as a lower molecular weight homolog missing the first two residues (Met-Phe-) from the N-terminus (theoretical mass of 21579.66). It was assumed that this heterogeneity would have minimal effect on the structural and stability characteristics of pGH. Gdn HCl for biochemical applications was obtained from Mallinckrodt (Paris, KY). All other chemicals and reagents were of at least analytical grade quality, and water was obtained from a Milli-Q (Millipore, Bedford, MA) water purification system.

2.2. Solution preparation

Stability assessment of pGH was conducted at 37°C in 0.2 M Tris buffer, pH 9.0 containing Gdn HCl (0–6 M) at an initial pGH concentration of 1 mg/ml (45.45 μM). Stock solutions of pGH (filtered through 0.2 μm microcentrifuge filters at $1700 \times g$ for 5 min) and Gdn HCl were each prepared in buffer and mixed to obtain the desired concentrations of each component. The actual concentration of Gdn HCl in the stock solution was determined by refractometry (Nozaki, 1972), and the pGH stock solution concentration was determined by UV absorbance at 278 nm using an absorptivity of 0.71 ml/mg per cm (15471 M^{-1}/cm) (Bastiras and Wallace, 1992). The final pH of each solution was checked and adjusted to pH 9 with dilute NaOH, if necessary.

2.3. Circular dichroism spectropolarimetry

CD measurements were made using a JASCO J710 spectropolarimeter (Japan Spectroscopic, Tokyo) calibrated with ammonium *d*-camphorsulfonate. Each spectrum represented the average of four accumulations obtained in 0.1 nm increments at a scan rate of 20 nm/min with a 1 s response time and a 1 nm band width. Samples were placed in water-jacketed, stoppered, quartz cuvettes (path lengths of 0.2 mm or 1 cm), and the sample temperature was controlled using a Neslab RTE 111 (Newington, NH) recirculating bath. Cell temperatures were calibrated using a thermistor probe placed directly in the sample cell, and wavelength scans were acquired following a 15 min equilibration at 37°C. Mean residue elipticity values were calculated using a mean residue molecular weight of 115.18.

2.4. Size exclusion chromatography

Size-based separation was performed using a Superose 12 column (Pharmacia, Uppsala, Sweden) and a Beckman isocratic HPLC (Model 116 pump and Model 167 UV detector, Beckman Instruments, San Ramon, CA). The mobile phase consisted of 0.1 M ammonium bicarbonate buffer, pH 8.5, containing Gdn HCl (at the same concentration as the sample) pumped at a flow rate of 0.5 ml/min. A 50 μl aliquot of each sample was injected onto the column and UV detection was conducted at 278 nm.

2.5. SDS-PAGE

SDS-protein complexes were formed by incubating the protein (pGH or molecular weight standards) with 2.5% (w/v) SDS at 90°C for 15 min under either reducing (10% v/v β-mercaptoethanol) or non-reducing conditions. pGH (approximately 1 μg) was applied to gradient 8–25% gels (Pharmacia) and separation was performed using a Phast System (Pharmacia) according to the manufacturers instructions. Protein mobility was compared with protein standards (Pharmacia) of known molecular weights ranging from 14.4 to 94 kD.

2.6. Reversed phase HPLC

RP-HPLC was conducted using an Aquapore RP300 (220×4.6 mm, i.d.) C8 column (Perkin–Elmer, Norwalk, CT) and a Beckman gradient HPLC system (Model 126 pump, Model 167 UV detector and an Altex injector). The mobile phase comprised 0.1% aqueous trifluoroacetic acid (TFA) (solvent A) and 0.1% TFA in 70% acetonitrile (ACN) (solvent B) and was pumped at a flow rate of 1.0 ml/min. The percentage of solvent B was varied form 64 to 92.3% over 17 min (1.32%/min B), and the column temperature was maintained at 40°C. The injection volume was 20 μl and UV detection was conducted at 220 nm.

2.7. Peptide mapping

Characterization of the sites of modification in pGH was conducted using a previously described peptide mapping procedure (Charman et al., 1993a). Briefly, pGH samples were digested with trypsin (using an enzyme:substrate ratio of 1:20 w/w) for 20 h at 4°C, and separation of the resulting tryptic peptides was achieved using gradient HPLC with UV detection at 220 nm.

2.8. Data analysis

Integrated peak areas for individual peaks from the RP-HPLC analyses were expressed relative to the total peak area in the same chromatogram to minimize variability associated with slight volume changes (e.g. different injection volumes or minor solvent evaporation). The total peak area remained within $\pm 10\%$ for the duration of the experiment verifying that all degradation products were detected at 220 nm and that significant protein precipitation did not occur over the sampling period. The relative peak area for native pGH (Met-pGH + des Met-Phe- homolog) was plotted as a function of time and the pseudo first-order degradation rate constant and the standard error of the estimate were determined by non-linear curve fitting.

3. Results and discussion

3.1. Effect of Gdn HCl on the conformation of pGH

The far-UV CD spectrum of pGH at pH 9.0 and 37°C was characterized by minima at 222 and 209 nm which is typical for proteins with a high degree of α-helix content. Previous investigators have reported the helical content of pGH to be approximately 50% (Abdel-Meguid et al., 1987). Incubation of pGH under the same conditions but with increasing concentrations of Gdn HCl showed a progressive decrease in the CD signal between 250 and 210 nm indicating loss of secondary structure (Fig. 1A). Due to the background absorbance of Gdn HCl below 210 nm, the secondary structure content of pGH in the presence of denaturant could not be assessed.

The near-UV CD spectrum for native pGH was characterized by absorption minima at 263, 268, 286 and 292 nm resulting from Phe and Tyr residues and the single Trp residue (Fig. 1B). At intermediate (3 and 4 M) concentrations of Gdn HCl, there was an decrease in the intensity of the CD band at 286 nm and a corresponding increase in the intensity of a new band at 300 nm. In 3 M Gdn HCl, the mean residue elipticity at 300 nm was also found to be concentration dependent (data not shown). In the presence of 5 and 6 M Gdn HCl, the near-UV CD spectra (Fig. 1B) of pGH was essentially featureless except for weak bands between 250 and 275 nm indicating the absence of significant tertiary structure under these conditions.

SEC was used to monitor size related changes in pGH in the presence of Gdn HCl (Table 1). The elution volume of native pGH was 13.7 ml which decreased to 11.6 ml in the presence of 6 M Gdn HCl consistent with an increase in the hydrodynamic radius upon unfolding. In the presence of 3 M Gdn HCl, two peaks were observed with elution volumes of 10.2 and 13.3 ml. The elution volume of the earlier peak was less than that observed for fully denatured pGH (6 M Gdn HCl), suggesting that it represented an associated form. In 4 M Gdn HCl, a single peak with an elution volume of 12 ml was detected by SEC.

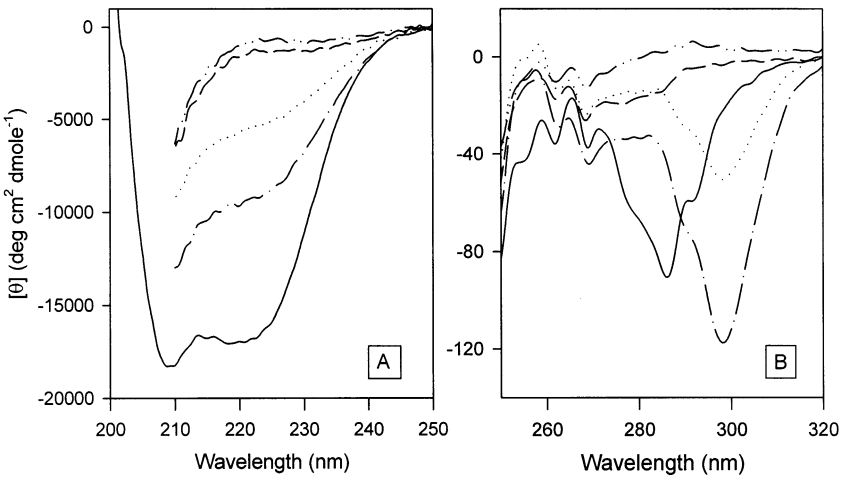

Fig. 1. Far-UV CD spectra (A) and near-UV CD spectra (B) of pGH (1 mg/ml) with varying concentrations of Gdn HCl. The pGH solutions were prepared in 0.2 M Tris buffer, pH 9, containing 0 M (——), 3 M (– · –), 4 M (· · ·), 5 M (– – –) and 6 M (– · · –) Gdn HCl, and the spectra were recorded at 37°C.

This would appear inconsistent with the near UV CD data which showed evidence of the associated form being present in 4 M Gdn HCl. However, since SEC is a diluting technique, the absence of an associated peak is likely to be due to dissociation following injection. The elution volume for pGH in 5 M Gdn HCl was similar to that in 6 M Gdn HCl.

The CD and SEC data reported here are consistent with reports of the Gdn HCl denaturation characteristics of both bGH (Brems et al., 1986; Havel et al., 1986), which is 91% homologous with pGH, and pGH (Bastiras and Wallace, 1992). Each of these reports described the population of a self-associated species at intermediate concentrations of Gdn HCl (3–4 M) which was characterized as a having a hydrodynamic Stokes' radius greater than either the native or the denatured protein. Under conditions where the associated form of bGH was populated, the corresponding near UV CD signal exhibited an intense negative CD band at 300 nm which was concentration dependent and was not present for either the folded (0 M Gdn HCl) or unfolded (6 M Gdn HCl) protein (Havel et al., 1986). Based upon studies with the bGH peptide fragment corresponding to residues 96–133, the association was proposed to result from interaction between the exposed hydrophobic residues of the amphiphilic helix 3 (residues 110–127) (Brems et al., 1986). Given the similarity in the CD and SEC results for pGH described here and elsewhere (Bastiras and Wallace, 1992) relative to those observed for bGH and the structural homology between pGH and bGH (the third helices are identical except for a conservative Leu → Gln substitution at residue 121 in pGH), it is likely that self-association of pGH occurs in a manner similar to that described for bGH.

Table 1
SEC elution characteristics for pGH and degradation products following incubation in 0.2 M Tris buffer, pH 9 and 37°C in the absence and presence of Gdn HCl

[Gdn HCl] (M)	Elution volume (ml)	
	Initial ($t = 0$ h)	Degraded sample
0	13.7	12.5, 13.7 ($t = 191$ h)
3	10.2, 13.3	7.7, 8.9, 10.2 ($t = 70$ h)
4	12.0	—
5	11.8	—
6	11.6	9.9, 11.2, 11.6 ($t = 113$ h)

The incubation times for the degraded samples are shown in parentheses.

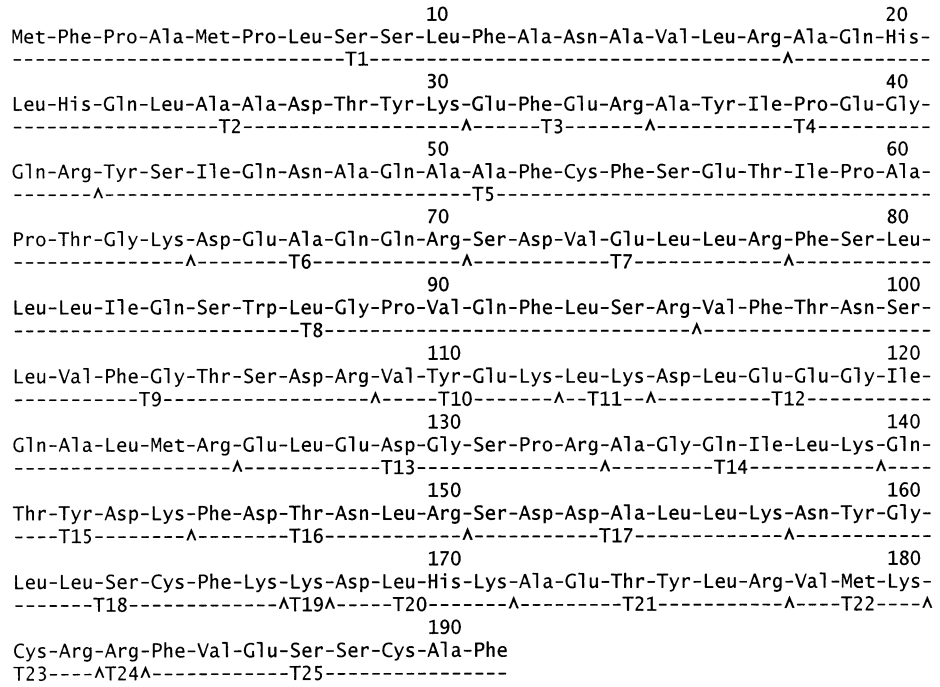

Fig. 2. Amino acid sequence of Met-pGH showing the predicted sites of trypsin cleavage.

3.2. Degradation of pGH in buffer

Samples of pGH incubated in pH 9.0 buffer at 37°C for up to 200 h were analyzed by peptide mapping, RP-HPLC, SEC and SDS-PAGE in an attempt to qualitatively assess the primary sites of degradation. As the focus of the study was the effect of solvent conditions (and corresponding conformational changes) on degradation kinetics, conclusive identification of degradation products was not attempted. Tentative assignments were made based on comparison with previously reported studies.

3.2.1. Peptide mapping

Fig. 2 illustrates the amino acid sequence of pGH and the predicted sites for trypsin cleavage. As depicted in Fig. 3, the primary sites of chemical modification following incubation for 336 h were in tryptic fragments T9 (corresponding to residues 96–108 containing Asn^{99}), T23–T25 (residues 181–182 linked by the 'small loop' disulphide bond to residues 184–191), and T5–T18 (residues 43–64 linked by the 'large loop' disulphide bond to residues 158–166). Disappearance of the peak corresponding to fragment T9 was accompanied by the formation of two new peaks (labelled b and c in Fig. 3B) which formed in an approximate ratio of 3:1. Amino acid analysis indicated peaks b and c had the same composition as the T9 fragment, however this analysis would not distinguish between Asn and Asp residues. Subsequent studies in which the parent T9 fragment and peaks b and c were isolated and analyzed by FAB-MS indicated that the mass of each degradation peak was one unit greater than T9 indicating that they represented deamidated forms (Asp^{99} and $isoAsp^{99}$). Digestion of peaks b and c with *Staphylococcus aureus* V8 protease, which cleaves at the carboxyl side of Glu and Asp residues under the conditions employed (Wilkinson, 1986), confirmed peak c to be the Asp^{99} variant and peak b the $isoAsp^{99}$ variant (unpublished data).

Formation of $isoAsp^{99}$ and Asp^{99} degradation products of pGH in similar ratios (3:1

isoAsp[99]:Asp[99]) following incubation at alkaline pH has previously been reported (Violand et al., 1990; Hageman et al., 1992) and is consistent with deamidation occurring via a succinimide intermediate arising from the β-aspartyl shift mechanism (Bornstein and Balian, 1977; Geiger and Clarke, 1987). The formation of succinimide intermediates is typically facilitated when Asn residues are in regions of random structure enabling attainment of correct bond angles and group orientations. The Asn[99] residue in pGH is situated in the connecting loop between helices 2 and 3 which is in a very flexible region of the polypeptide chain (Violand et al., 1990). In comparison, Asn[99] in human growth hormone (hGH) is located in a minor helix encompassing residues 94–100 (De Vos et al., 1992) and does not readily undergo deamidation (Johnson et al., 1989).

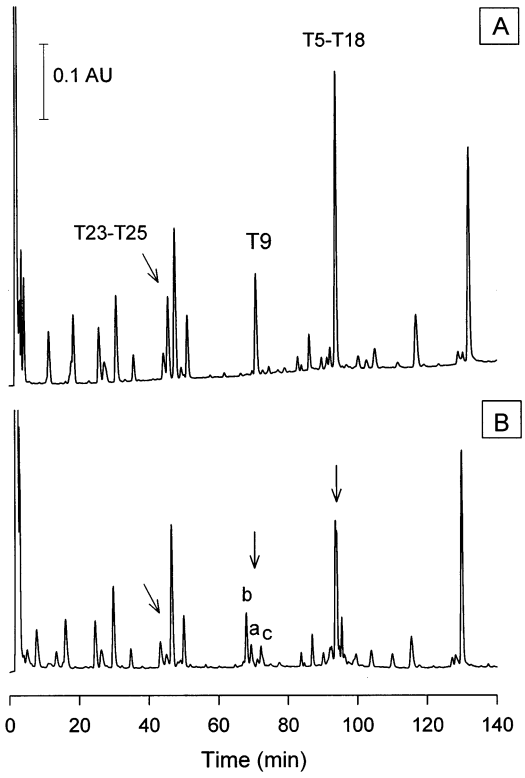

Fig. 3. Chromatograms of a digest of pGH following incubation in 0.2 M Tris buffer, pH 9 at 37°C for 0 h (A) and 336 h (B). The arrows in (B) indicate the peaks in the chromatogram that changed significantly upon incubation. The peaks designated 'a', 'b', and 'c' represent tryptic fragments T9, isoAsp[99]-T9, and Asp[99]-T9, respectively. Refer to Charman et al. (1993a) for other peak designations.

The loss of the peak corresponding to fragment T5–T18, which contains the 'large loop' disulphide bond and two Asn and two Gln residues, was associated with appearance of two additional peaks which were poorly resolved from the parent T5–T18 peak. Given the similarity in elution characteristics between the degradation products and parent peak, the products most likely represented deamidated species rather than crosslinked forms resulting from disulphide exchange reactions. Unfortunately, their poor resolution precluded attempts at isolation and further characterization of these degradation products.

The loss of the peak corresponding to fragment T23–T25 (containing the 'small loop' disulphide bond) was attributed to degradation of the disulphide bond linking Cys[181] and Cys[189]. This disulphide has been implicated in the formation of a reducible, covalent dimer following incubation of high concentrations of pGH at neutral pH and 40°C (Buckwalter et al., 1992). These same authors demonstrated that reduction and alkylation of Cys[181] and Cys[189] resulted in a significant improvement in the thermostability of pGH under the solution conditions employed.

3.2.2. Reversed phase HPLC

The RP-HPLC method for monitoring pGH was adapted from published methods which examined the degradation of native pGH and bGH (Violand et al., 1990; Hageman et al., 1992). Under these conditions, the isoAsp[99] derivative of pGH eluted prior to the main peak for native pGH, whereas the Asp[99] degradation product coeluted with the parent peak. A representative RP-HPLC chromatogram of pGH, presented in Fig. 4A, depicts two peaks which corresponded to native met-pGH (labelled N) and the des Met-Phe- homolog (labelled H). Incubation of pGH in pH 9 buffer at 37°C resulted in the formation of four new peaks (labelled 1–4 in Fig. 4B) with peak 1 being tentatively identified as the isoAsp[99] variant of pGH and peak 2 assigned to the isoAsp99 variant of the des Met-Phe- homolog.

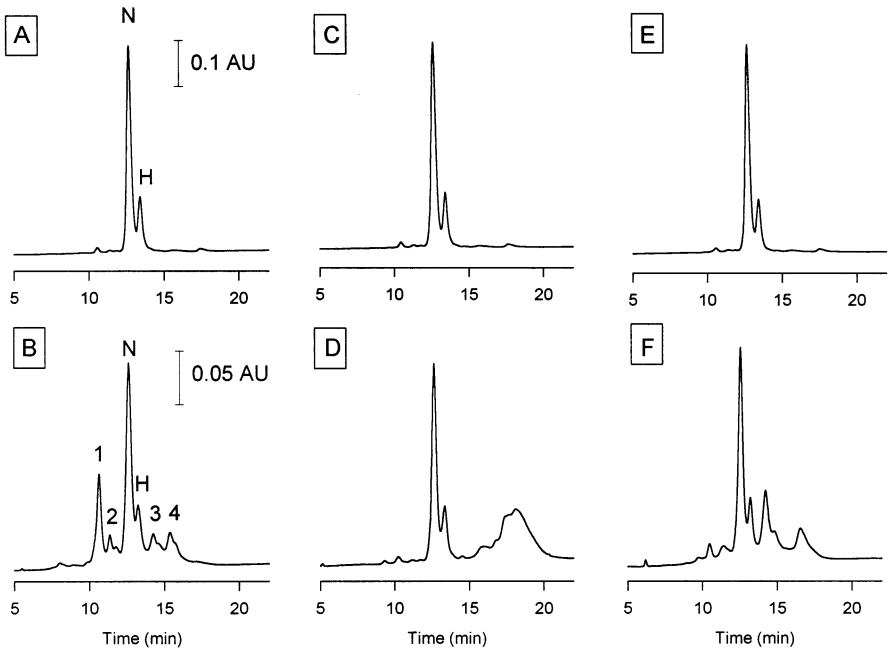

Fig. 4. RP-HPLC profiles of pGH (1 mg/ml) in 0.2 M Tris buffer, pH 9 (A) and (B) and the same buffer containing 3 M (C) and (D) and 6 M (E) and (F) Gdn HCl. Samples were incubated at 37°C for 120 h (buffer, (B)), 8 h (3 M Gdn HCl, (D)) and 24 h (6 M Gdn HCl, (f)). In (A) and (B), the peaks designated 'N' and 'H' refer to native Met-pGH and the des Met-Phe- homolog, respectively. Peaks 1 and 2 were tentatively assigned to the isoAsp[99] degradation products of N and H, respectively. The later-eluting peaks (peaks 3 and 4) were tentatively assigned as crosslinked degradation products of pGH.

3.2.3. Size exclusion chromatography

The major size related changes following incubation of pGH in the absence of denaturant was the formation of a high molecular weight species (elution volume 12.5 ml) with a corresponding decrease in peak area for monomeric pGH (Table 1 and Fig. 5A). SEC was conducted under non-denaturing conditions and would therefore detect high molecular weight species formed through either non-covalent or covalent interactions. In contrast, non-covalent aggregates of pGH would have been dissociated by the mobile phase used in the RP-HPLC assay. The high molecular weight peak was collected following elution from the SEC column and re-analyzed using the RP-HPLC assay. The reversed phase assay revealed two main peaks with retention times identical to those of peaks 3 and 4 in Fig. 4B leading to their tentative identification as high molecular weight crosslinked species. Under the analytical conditions employed, it was not possible to assess whether peaks 3 and 4 were also deamidated.

The relative amounts of non-covalent and covalent aggregates were assessed by comparison of the percentage of high molecular weight species detected by SEC and RP-HPLC (Table 2). The results indicated that a similar proportion of high molecular weight products was detected by each method over the time-course of the reaction, suggesting that they were primarily covalent in nature. Based upon SEC and RP-HPLC data, covalent high molecular weight products ac-

Table 2
Percentage of high molecular weight products detected by RP-HPLC and SEC during incubation of pGH in 0.2 M Tris buffer, pH 9 at 37°C

Incubation time (h)	% high M_W products "RP-HPLC"	% high M_W products "SEC"
0	0	0
49	12	13
99	21	23
191	26	33

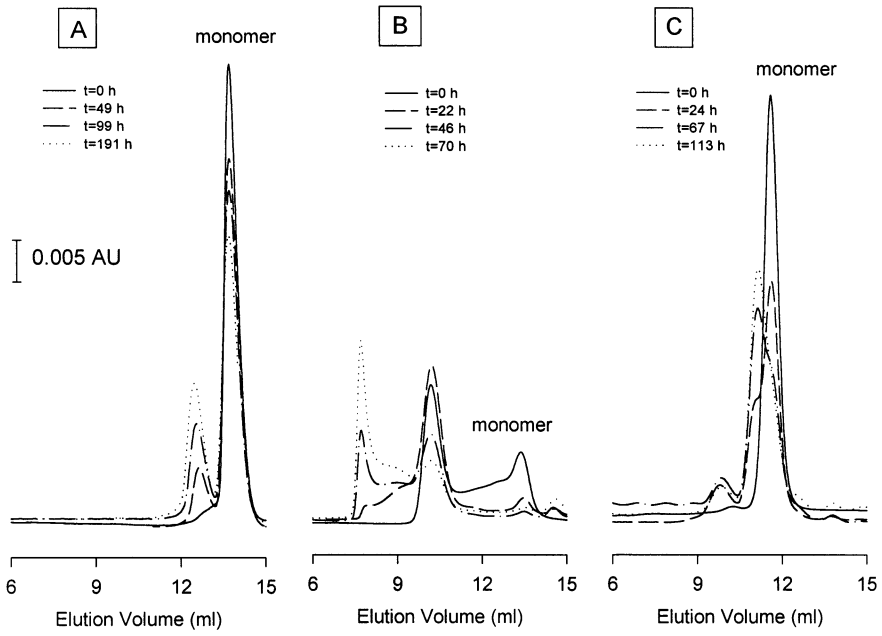

Fig. 5. SEC profiles of pGH (1 mg/ml) in 0.2 M Tris buffer, pH 9 (A), and the same buffer containing 3 M (B) or 6 M (c) Gdn HCl following incubation at 37°C.

counted for approximately 30% of the total peak area in the chromatogram after incubation at 37°C for 191 h.

3.2.4. SDS-PAGE

The high molecular weight products of a degraded pGH sample were further characterized by SDS-PAGE conducted under non-reducing and reducing conditions. Under non-reducing conditions, two bands were present corresponding to molecular weights of approximately 21 and 42 kD (data not shown). Under reducing conditions, the intensity of the 42 kD band decreased with a concomitant increase in the intensity of the band for native pGH at 21 kD. A degraded pGH sample was also reduced by the addition of excess dithiothreitol and the products solubilized by addition of Gdn HCl to a final concentration of 3.3 M. RP-HPLC analysis indicated partial disappearance of the late eluting peaks (peaks 3 and 4) with increases in the peaks corresponding to the native and deamidated species. The decreased intensity of the high molecular weight RP-HPLC peaks, and the diminution in the SDS-PAGE dimer band under reducing conditions, suggested that the dimeric products were formed, in part, from intermolecular disulphide crosslinking. The degradation pathways for pGH observed under non-denaturing conditions in this study are consistent with literature reports where deamidation and covalent crosslinking were identified following incubation at neutral to alkaline pH (Violand et al., 1990; Buckwalter et al., 1992; Hageman et al., 1992).

3.3. Degradation of pGH in the presence of Gdn HCl

Samples of pGH in pH 9 buffer containing Gdn HCl were incubated at 37°C and characterized by RP-HPLC and SEC. Sample analysis by peptide mapping and SDS-PAGE was precluded by the presence of Gdn HCl. Attempts to dialyze the Gdn HCl-containing solutions against buffer to allow for tryptic digestion were unsuccessful resulting in significant aggregation and precipitation.

3.3.1. Degradation of pGH in 3 and 4 M Gdn HCl

The RP-HPLC retention characteristics of pGH in the presence of Gdn HCl prior to incubation (Fig. 4C,E) were identical to those in the absence of Gdn HCl (Fig. 4A) indicating that the associated form of pGH present in the 3 and 4 M Gdn HCl buffers (see CD data in Fig. 1 and SEC data in Table 1) was completely dissociated by the RP-HPLC mobile phase. These data also confirmed that the rapid dilution of Gdn HCl following injection onto the HPLC column did not induce pGH precipitation as the peak areas for the zero-time samples in the absence and presence of Gdn HCl were equivalent.

The RP-HPLC chromatograms demonstrated the formation of a series of broad late-eluting peaks upon incubation of pGH in 3 M Gdn HCl for 8 h (Fig. 4D). Similar peaks were also detected when pGH was incubated in 4 M Gdn HCl (not shown). The series of peaks eluting between 16 and 19 min were different to those observed following degradation of pGH in buffer alone (peaks 3 and 4 in Fig. 4B), indicating that different products were formed under the partially denaturing conditions induced by 3 or 4 M Gdn HCl. The retention behaviour of these broad, late-eluting peaks was consistent with the formation of covalent oligomeric species. Following addition of excess dithiothreitol to samples which had been incubated for 24 h in either 3 or 4 M Gdn HCl, the area of the late-eluting peaks was reduced with a corresponding increase in peak area for monomeric pGH. Further incubation of pGH in 3 or 4 M Gdn HCl for greater than 100 h revealed a predominant late-eluting peak with a retention time of approximately 18 min. Reduction of this sample resulted in a decrease in the area for the 18 min peak with an increase in a peak corresponding to monomeric pGH.

SEC analysis of pGH incubated in 3 M Gdn HCl demonstrated the formation of oligomeric species with elution volumes of approximately 7.7 ml (equivalent to the total exclusion volume of the column) and 8.9 ml (Fig. 5 and Table 1). After 22 h incubation, monomeric pGH (elution volume 13.3 ml) was minimally detected when the samples were analyzed by SEC using the corresponding Gdn HCl mobile phase, but the RP-HPLC assay indicated the presence of monomeric pGH. These observations indicated that some of the high molecular weight products observed by SEC analysis were non-covalent in nature and disrupted by the denaturing RP-HPLC mobile phase.

3.3.2. Degradation of pGH in 5 and 6 M Gdn HCl

The degradation of pGH in pH 9.0 buffer containing 6 M Gdn HCl for 24 h resulted in the formation of late eluting peaks when samples were analyzed by RP-HPLC (Fig. 4F). The retention times of these late eluting peaks were similar to those for the dimeric species observed following degradation of pGH in buffer without Gdn HCl. Similar degradation peaks were detected when pGH was incubated in 5 M Gdn HCl (not shown). However, as characterization of the degradation products was not conducted due to difficulties in isolation, it cannot be assumed that the species formed in 5 and 6 M Gdn HCl were the same as those formed in buffer on the basis of the similar retention characteristics. The addition of excess dithiothreitol to pGH incubated in 6 M Gdn HCl for 24 h resulted in disappearance of the peak eluting at 16 min with a corresponding increase in the peaks for monomeric and deamidated pGH. Upon further incubation of pGH in 5 or 6 M Gdn HCl for periods greater than 100 h, the predominant degradation product detected by RP-HPLC eluted at 14 min. Reduction of this sample produced no significant change in the peak area indicating that disulphide exchange processes may have contributed to the formation of intermediate degradation products which underwent further reaction to form non-reducible covalent products.

SEC analysis of pGH incubated in 6 M Gdn HCl demonstrated the formation of two high molecular weight species eluting at approximately 9.9 and 11.2 ml (Fig. 5 and Table 1). These species were most likely formed through covalent interactions as the highly denaturing 6 M Gdn HCl SEC mobile phase would have dissociated non-covalent, hydrophobic aggregates. The elution characteristics indicated that the products were likely to be dimers in contrast to the

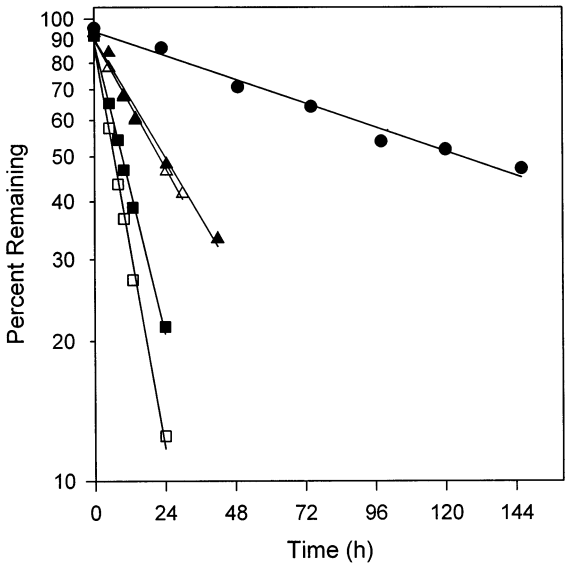

Fig. 6. Relative peak area versus time data for the RP-HPLC peak corresponding to pGH (N + H) following incubation in 0.2 M Tris buffer, pH 9 at 37°C. Solutions were prepared in buffer containing 0 M (●), 3 M (□), 4 M (■), 5 M (△) and 6 M (▲) Gdn HCl.

oligomeric products formed in 3 and 4 M Gdn HCl.

3.4. Comparative kinetics of pGH degradation

The loss of pGH incubated in buffer or varying Gdn HCl concentrations and monitored by RP-HPLC followed apparent first-order kinetics over the reaction period (Fig. 6). Peak area data for highly degraded samples (incubated in buffer for greater than 145 h, or 5 and 6 M Gdn HCl for greater than 50 h) were omitted due to inadequate peak resolution and inaccurate integration. The integrated area for peaks corresponding to native Met-pGH (peak N in Fig. 4A) and the des Met-Phe- homolog (peak H in Fig. 4A) were combined to obtain the kinetic data however, similar rate constants were obtained using either combined areas (peaks N + H) or the area for peak N only. Table 3 presents the estimated degradation rate constants and standard errors for the estimates and half-lives when pGH was incubated under the different solution conditions.

The stability of pGH was highly dependent upon the concentration of Gdn HCl present in solution with the intermediate Gdn HCl solutions being the least stable. The notable conformational feature of pGH in 3 or 4 M Gdn HCl was the initial presence of the non-covalent associated species, as evidenced by the high molecular weight species detected by SEC (Table 1) and the corresponding negative near-UV CD band at 300 nm (Fig. 1B) which was concentration dependent. In the presence of Gdn HCl, the decrease in pGH stability relative to a buffer control was associated with an increase in the formation of oligomeric species which were largely covalent in nature. While this may not be surprising given that aggregation (both covalent and non-covalent) generally increases with denaturation, the striking difference in the rates of degradation at intermediate and high Gdn HCl concentrations was not predicted.

The apparent increased degradation rate and the corresponding formation of oligomeric products in the presence of Gdn HCl is consistent with decreased stability of the disulphides in the unfolded protein relative to the native form. Analysis of the crystal structure of pGH has indicated that the 'small loop' disulphide is located near the C-terminus and is exposed to the external solvent environment (Abdel-Meguid et al., 1987). Previous studies have shown that the 'small loop' disulphide linking Cys^{181} and Cys^{189} is selectively alkylated following reduction of pGH with dithiothreitol providing evidence that the 'large loop' disulphide is at least partially buried under these solution conditions (Buckwalter et al., 1992). It is

Table 3
Effect of Gdn HCl on the observed pseudo first order rate constant for degradation of pGH (1 mg/ml) in 0.2 M Tris buffer, pH 9 at 37°C

[Gdn HCl] (M)	k_{obs} (h^{-1}) $\times 10^3$ ($\pm$S.E.)	Apparent $t_{1/2}$ (h)
0	5.2 ($\pm$0.3)	133.2
3	92.7 ($\pm$2.4)	7.5
4	64.5 ($\pm$1.8)	10.7
5	27.8 ($\pm$1.3)	24.9
6	26.6 ($\pm$2.0)	26.1

reasonable that the conformational changes incurred in the presence of Gdn HCl resulted in an increased exposure of the 'large loop' disulphide which may have resulted in an increase in reactivity.

The most obvious difference in pGH solutions containing 3 or 6 M Gdn HCl which could account for the unexpected differences in degradation rates is the difference in conformation. Spectroscopic and SEC studies reported here and elsewhere (Bastiras and Wallace, 1992) have shown that in intermediate Gdn HCl concentrations (approximately 2.8–3.5 M), pGH exists in a conformational state that has a high tendency to self-associate. The reported fluorescence and CD results are consistent with exposure of the single tryptophan residue normally present in a hydrophobic pocket under these solution conditions. Previous work reported by our laboratory also indicated that the species formed in intermediate concentrations of Gdn HCl has a high tendency to precipitate upon rapid dilution of the Gdn HCl into a non-denaturing, aqueous buffer (Charman et al., 1993b). This trend was not evident for pGH prepared in either buffer or 6 M Gdn HCl. Whether or not these conformational differences between 3 and 6 M Gdn HCl solutions of pGH are responsible for the differences in reactivity can only be speculated at this stage.

The presence of Gdn HCl would have clearly resulted in a significant increase in the ionic strength and it is not possible to rule out an ionic strength contribution to changes in reaction rates. However, in studies conducted by Volkin and Klibanov (1987), the time course for the destruction of disulphide bonds in insulin at 100°C and pH 8.0 was shown to be similar in the absence and presence of 6 M Gdn HCl suggesting that once the tertiary structure was disrupted (i.e. minimal structure would have been present at 100°C), the effect of Gdn HCl on the reactivity of the disulphides was minimal. Reports in the literature regarding the effect of ionic strength on rates of deamidation are somewhat contradictory and have primarily been conducted using small model peptides with the ionic strength being controlled with NaCl. One report by Tyler-Cross and Schirch (1991) indicated that concentrations of up to 1 M Gdn HCl or 1 M LiCl had minimal effect on the rate of deamidation of a pentapeptide at pH 8.0. Being a neutral molecule, urea would have eliminated the potential ionic strength effect but was not used in these investigations due to inadequate stability under long term storage at alkaline pH (Hagel et al., 1971).

Unfortunately, the effect of these solution conditions on the extent of deamidation could not be assessed using the described analytical methods due to the predominance of intermolecular reactions. The use of ion exchange chromatography was considered as a means of gaining insight into the possible differences in deamidation rates, however it is likely that this form of chromatography would have also been sensitive to the charge differences of monomeric versus oligomeric species making the interpretation of results difficult, if not impossible. Other specific techniques to assess deamidation, such as the commercial assay kit marketed by Promega. which detects the formation of isoaspartic acid (Isoquant™ protein deamidation detection kit, Promega, 1995), could not be utilized due to the presence of Gdn HCl.

4. Conclusions

This report demonstrated that solution conditions had a significant influence on the rate of degradation of pGH, with unexpected findings being the significantly enhanced reactivity of pGH at intermediate concentrations of Gdn HCl and the apparent differences in product distribution between buffer, intermediate and high Gdn HCl concentrations. While it cannot be concluded that differences in reaction rates were linked to conformational changes in pGH or the presence of associated species in 3–4 M Gdn HCl, the results clearly indicate that chemical reactivity is strongly influenced by solution conditions in a manner which may not be immediately obvious or predictable. These findings have relevance in the choice of conditions used to isolate, process and formulate proteins. Given that chemical instability may be accelerated under denaturing conditions often required to solubilize proteins in inclusion bodies, it is essential that conditions are

rationally chosen to minimize the rate of potential chemical reactions.

The potential effect of protein conformation on the rate and mechanisms of degradation must be considered during the design and interpretation of accelerated stability studies, particularly in the presence of excipients whose effects on conformation may be largely unknown. Typically, accelerated stability studies of proteins are restricted to temperatures below 40°C in order to limit the effect of temperature-induced conformational changes on non-covalent aggregation (Pearlman and Nguyen, 1992). The absence of conformational changes at the chosen elevated temperature should be confirmed in the presence of formulation excipients as the effects of these components may not be evident at room temperature.

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