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High-performance liquid chromatography of amino acids, peptides and proteins

XCIX[a]. Comparative study of the equilibrium refolding of bovine, porcine and human growth hormone by size-exclusion chromatography

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ABSTRACT

The equilibrium refolding of bovine, porcine and human growth hormone and ovine prolactin in guanidine hydrochloride has been investigated using high-performance size-exclusion chromatography (HPSEC). It was found that bovine and porcine growth hormones exhibited very similar refolding behaviour. However, the renaturation of human growth hormone followed a different pathway. In particular, the folding transition of human growth hormone occured at 4.7 *M* guanidine hydrochloride compared to 3.8 and 3.5 *M* for the bovine and porcine molecules, respectively, and 3.5 *M* for ovine prolactin. The refolding mechanism of an internally clipped fragment derived from partial tryptic digestion, exhibited similar folding properties to the corresponding intact molecule. The internally clipped analogue existed as a relatively larger molecule under fully denaturing conditions. Reduction followed by carboxymethylation resulted in growth hormone molecules with significantly reduced stability and altered folding properties. The results have been correlated with differences in structure to further demonstrate the utility of HPSEC in the study of protein folding and stability.

INTRODUCTION

The mechanism by which nascent polypeptide chains adopt their native three-dimensional structure remains a fundamental problem in biology. It is generally considered[1,2] that the protein folding process involves the early formation of

[a] For part XCVIII, see ref. 27.

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hydrogen-bonded secondary structures associated with specific nucleation sequences. These events are then followed by reorganisation to yield tightly packed domains constituting the tertiary structure. However, the precise role of the primary amino acid sequence in directing protein folding pathways is not yet understood. As a consequence, the current state of understanding does not allow tertiary structures to be predicted *de novo* solely from the knowledge of the primary amino acid sequence, although useful predictive algorithms which indicate preferred regions of α- or 3_{10}-helical content, β-sheet, or β-turn structures have found wide application over the past decades[3,4].

The reversible denaturation/renaturation pathways of proteins in bulk solution have generally been studied using a combination of techniques including ultraviolet and fluorescence spectroscopy, two-dimensional nuclear overhauser spectroscopy, circular dichroism and sedimentation velocity measurements. These approaches allow the detection and characterisation of specific equilibrium intermediates which are formed during the folding process. In addition, comparison of the unfolding pathways of closely-related proteins with modified sequences derived from different species[5,6] or through recombinant DNA site-directed mutagenesis[7] has been used to provide further characterization of specific amino acid residues which influence protein conformation.

Size-exclusion chromatography (SEC) has also been utilised as an experimental tool for studying protein folding pathways[8–10]. Recent advances in the development of column packing materials for SEC have resulted in higher levels of performance in analytical systems in terms of resolution and separation speed. As a result, high-performance SEC (HPSEC) methods can now be used to monitor relatively minor changes in protein hydrodynamic volumes, apparent equilibrium constants and Gibbs free energy of stabilisation associated with the unfolding or refolding of a protein under different conditions.

In the present study, HPSEC has been used to investigate the equilibrium renaturation pathway in guanidine hydrochloride (Gdn · HCl) of a series of growth hormones (GHs), namely porcine (pGH), bovine (bGH) and human (hGH) and the ovine lactogenic protein, prolactin (oPrl). These proteins are members of the somatotrophic family of structurally conserved pituitary and placental proteins involved in the regulation of growth and lactogenesis in mammals. These proteins are comprised of approximately 190 amino acid residues with molecular weights of about 22 000. In addition to their well-established growth-promoting activity, the GHs are also known to exhibit several other important physiological properties which include significant effects on protein, lipid and carbohydrate metabolism. For example, within the GH molecule, the somatotrophic, lactogenic, diabetogenic and insulin-like action are mediated by specific regions defined by both the topographical and primary structural features. The three-dimensional low resolution 2.8 Å X-ray crystal structure of recombinant pGH has been reported[11] and indicates the presence of four anti-parallel amphipathic helices arranged in a left twisted helical bundle. Alignment of the amino acid sequence of pGH with that of other growth hormones reveals that the residues located within the α-helices are predominantly invariant[12]. This observation suggests that these amino acid residues are necessary for the maintenance of structural integrity of these proteins. The present paper presents results on the refolding behaviour of bGH, pGH, hGH and oPrl and provides insight into the

relationship between amino acid sequence, the mechanism of protein folding and the conformational stability of this protein superfamily.

MATERIALS AND METHODS

Chemicals and reagents

Water was quartz distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Acetonitrile (HPLC grade) was obtained from Mallinckrodt (Paris, KE, U.S.A.) and trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, U.S.A.). Gdn · HCl (enzyme grade) was purchased from Bethesda Research Labs. (Gaithersburg, MD, U.S.A.). Human prolactin, recombinant bovine growth hormone (r-bGH) and recombinant porcine growth hormone (r-pGH) were available through associated studies with the Centre for Bioprocess Technology whilst the pituitary derived human growth hormone was kindly provided by Commonwealth Serum Laboratories (Melbourne, Australia). Automated Edman sequence analysis on the carboxymethylated proteins and derived enzymic fragments has confirmed the sequence veracity of these proteins. Dithiothreitol, iodoacetic acid and L-1-Tosylamide-2-phenylethylchloromethyl ketone (TPCK) trypsin were purchased from Sigma (St. Louis, MO, U.S.A.), Fluka (Buchs, Switzerland) and Worthington (Freehold, NJ, U.S.A.), respectively.

Partial tryptic digests

Digestion was carried out by the addition of TPCK trypsin (1%, w/w, in 1 m*M* hydrochloric acid) to a solution of growth hormone (1 mg/ml in 0.15 *M* sodium chloride, 0.05 *M* Na_2HPO_4, pH 8.0) and the mixture was incubated for 6 min at room temperature. Digestion was stopped by acidification with 2 *M* hydrochloric acid.

Reductive alkylation

Dithiothreitol [300 μg in 30 μl of 200 m*M* trishydroxymethylaminomethane (Tris) (pH 8.0) containing 6 *M* Gdn · HCl and 2 m*M* EDTA] was added to pre-incubated solutions (30 min at 37°C) of growth hormone and the mixture was heated at 100°C for 5 min. After cooling, iodoacetic acid (600 μg in 60 μl Tris · HCl, pH 7.9) was added and the sample was incubated in the dark for 15 min, after which 10 μl 2-mercaptoethanol was added.

HPSEC

All HPSEC experiments were performed with a Pharmacia (Uppsala, Sweden) fast protein liquid chromatographic (FPLC) system consisting of two P-500 syringe pumps, a V-7 injector coupled to a Waters M450 variable-wavelength UV detector (Waters Assoc., Milford, MA, U.S.A.). All measurements were routinely monitored at 215 nm and recorded using a Perkin-Elmer (Norwalk, CT, U.S.A.) LCI-100 integrator.

Isocratic elution was controlled with a Pharmacia GP250 solvent programmer, using Pharmacia Superose 12 columns (300 × 10 mm I.D.). All injections were made with SGE (Melbourne, Australia) syringes, and pH measurements were performed with an Orion (MA, U.S.A.) SA520 meter, equipped with a combination glass electrode.

The Superose 12 columns were pre-equilibrated at each guanidine concentration by mixing the appropriate proportions of buffers A and B which both contained 0.01 *M* Tris at pH 8.0 with buffer B also containing 6 *M* Gdn · HCl. Chromatography was carried out isocratically at 0.4 ml/min. The protein molecular weight calibration curve was obtained in 1 *M* Gdn · HCl using cytochrome *c* (11 000 dalton), ribonuclease (13 700 dalton), sperm whale myoglobin (17 000 dalton), ovine growth hormone (22 000 dalton), human carbonic anhydrase (29 000 dalton) and bovine serum albumin (67 000 dalton). V_o and V_t were determined using thyroglobulin (670 dalton) and sodium azide, respectively. Distribution coefficient (K_d) values were determined using the relationship $K_d = (V_e - V_o)/(V_t - V_o)$, see also eqn. 1. Growth hormone samples were dissolved in 100% buffer B at concentrations of 300 μg/ml unless otherwise specified. All solutions were filtered through a 0.45-μm filter prior to injection.

Characterization of growth hormone derivatives

Following partial tryptic digestion and/or reductive alkylation, all derivatives were purified by reversed-phase chromatography on a Waters Assoc. liquid chromatograph consisting of two Model 6000A solvent delivery pumps, a U6K universal injector, and a M660 gradient programmer. Detection was carried out using a Lambda-Max Model 481LC variable-wavelength monitor operating at 215 nm, coupled to a Model M730 data module.

Purification was carried out with a Bakerbond widepore butylsilica stationary phase (Baker, Phillipsburg, NJ, U.S.A.) with nominal particle diameter of 5 μm and average pore size of 30 nm packed into a 25 cm × 0.46 mm I.D. column). Mobile phases TFA–water (0.1:100, v/v) (buffer A) and TFA–water–acetonitrile (0.1:25:75, v/v/v) (buffer B). Elution was carried out using a linear gradient from 100% buffer A to 100% buffer B over 60 min at a flow-rate of 1 ml/min.

The volume of HPLC fractions was reduced on a Savant (Hicksville, NY, U.S.A.) SpeedVac Concentrator at ambient temperature. Automated Edman amino acid sequencing of the derivatives was carried out on an Applied Biosystems (Foster City, CA, U.S.A.) Model 470A Sequencer.

RESULTS AND DISCUSSION

Equilibrium renaturation of growth hormones and prolactin

Under ideal conditions of gel filtration when the sample volume is negligible compared to the chromatographic bed volume and where there is no interaction between the protein solute and the stationary phase, solute elution is characterised by a distribution coefficient, K_d, according to the relationship

$$K_d = \frac{V_e - V_o}{V_t - V_o} \tag{1}$$

where V_e is the solute elution volume. The term V_o is the occluded volume represented by elution volume of molecules totally excluded from entering the largest pores in the gel, and V_t represents the elution volume of a solute which will distribute freely between the mobile phase and all of the pores of the stationary phases. Thus, in the

absence of interactive effects, the only factors which contribute to the elution behaviour of solute molecules are steric effects based on the hydrodynamic size and shape of the solute.

Geometrically, a macromolecule can be described by the axial ratio as a measure of its asymmetry, its volume which is a function of partial specific volume and molecular weight, and the degree of hydration. Formal relationships between a number of specific geometrical factors and HPSEC elution data have been derived[13,14] in the development of universal calibration methods for HPSEC columns. For example, the solution properties of proteins can be characterised by the dependence of K_d on molecular weight, Stokes radius, or an intrinsic viscosity radius. According to Porath[15], the K_d value for a spherical or ellipsoid protein is proportional to the radius of gyration, R, of a hypothetical sphere of equivalent hydrodynamic properties to that manifested by the protein. The dependence of K_d on R can thus be described according to the relationship

$$K_d^{1/3} = a - bR \tag{2}$$

As the volume of a sphere is linearly related to R^3, the chromatographic K_d value for a particular protein is therefore proportional to its hydrodynamic volume, V. HPSEC has been shown to be a considerably more rapid technique for the study of the hydrodynamic properties of proteins under a wide range of solvent conditions than other transport techniques such as intrinsic viscosity measurements and sedimentation techniques. In particular, the ability to characterise solvent and temperature induced conformational changes in protein structure in terms of the degree of associated molecular expansion has provided significant insight into the unfolding pathways of several proteins[8,10]. It should be noted that the derivation of eqns. 1 and 2 assumes a two-state model for the chromatographic process and is not related to the protein refolding process which will be approximated by a more complex model.

Several recent studies[10,16,17] on the equilibrium denaturation of bovine growth hormone (bGH) have utilised HPSEC as a probe for changes in the molecular volume during unfolding of this protein in Gdn · HCl and urea. The non-coincidence of the denaturation transitions as detected chromatographically and spectroscopically, indicated the presence of several unfolded intermediates. Furthermore, while the native and fully unfolded species of bGH are monomeric, the intermediate unfolded forms were found to exist as both monomeric and associated structures. In the present study, similar methods have been extended to the equilibrium refolding behaviour from the fully unfolded state, of different members of the somatotrophic family of proteins. To characterise the refolding behaviour of these growth hormones, experimental studies have been carried out with bovine, porcine and human growth hormone in Gdn · HCl at different concentrations using a Superose 12 column. In particular, the Gdn · HCl concentration was varied between 1 and 6 M in solvents containing 0.1 M Tris buffered at pH 8.0. A typical molecular calibration curve of proteins of different molecular weight separated on the Superose 12 support obtained in 1 M Gdn · HCl is shown in Fig 1. It can be seen from the data that a linear relationship between K_d and log molecular weight (MW) was observed with this elution system.

The chromatographic profiles of bGH eluted under different concentrations of

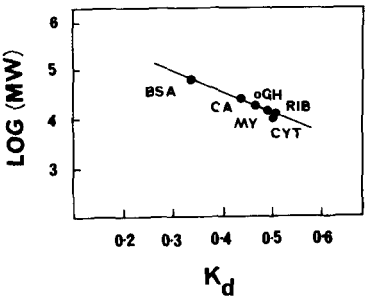

Fig. 1. Protein molecular weight (MW) calibration curve for the Superose 12 column in 1 *M* Gdn · HCl plus 0.1 *M* Tris. Proteins used are listed in the Materials and Methods section. The correlation coefficient, r^2, for the linear regresion analysis was 0.971. BSA = Bovine serum albumin, CA = human carbonic anhydrase, oGH = ovine growth hormone, MY = sperm whale myoglobin, RIB = ribonuclease and CYT = cytochrome *c*.

Gdn · HCl are shown in Fig. 2. Protein solutions were unfolded in 6 *M* Gdn · HCl and then loaded onto the column which was pre-equilibrated at different Gdn · HCl concentrations. While reasonably symmetrical peaks were observed for bGH at each Gdn · HCl concentration examined, the renaturation transition was clearly apparent between 3–4 *M* Gdn · HCl where there was a singnificant change in both the elution volume and the bandwidth of the solute peak. As employed previously, the parameters

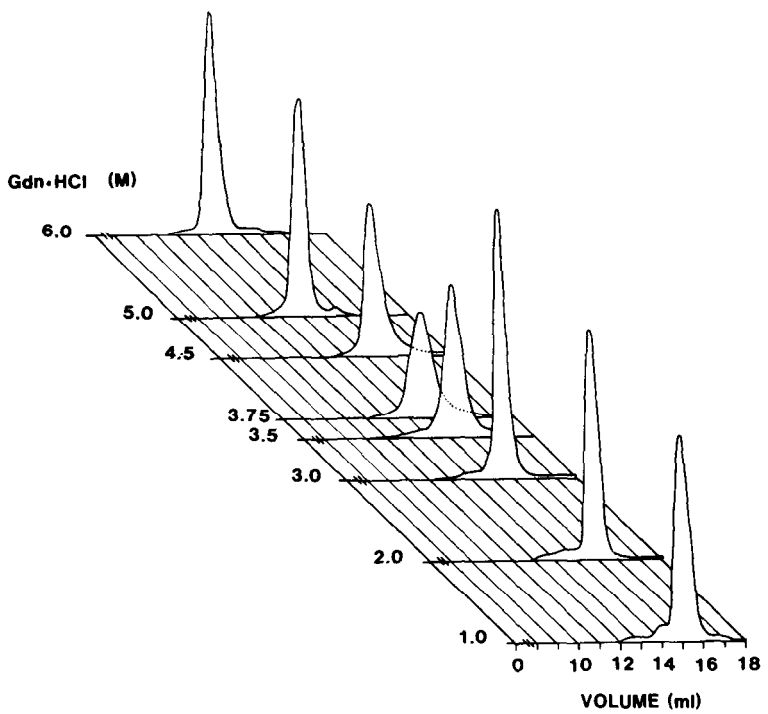

Fig. 2. Chromatographic elution profile of bGH as a function of Gdn · HCl concentration using HPSEC.

most useful to monitor the equilibrium refolding of the bGH molecules were the changes in apparent molecular volume relative to the native conformation and changes in the chromatographic peakwidth calculated at half peak height ($PW_{1/2}$) or from the second centralised moment for grossly asymmetric peaks. Since the kinetics of mass transport in a chromatographic sense will be similar for the different GH proteins, we have for the purposes of the present analysis used the $PW_{1/2}$ as a useful parameter to monitor the refolding pathway. The renaturation curves for bGH as monitored by these parameters are shown in Fig. 3a and b, respectively. In Fig. 3a, the relative change in K_d is represented by the ratio

$$\tau_D = \frac{K_{d,refolded}}{K_{d,native}} \tag{3}$$

where $K_{d,refolded}$ is the distribution coefficient of the refolded form at a particular Gdn · HCl concentration while $K_{d,native}$ is the distribution coefficient of bGH in 1 *M* Gdn · HCl, and is equivalent to the native conformation in neat buffer. The midpoint of refolding for bGH which is listed in Table I occurred at 3.8 *M* Gdn · HCl, while the τ_D ratio at 6 *M* Gdn · HCl corresponded to a 3.5-fold expansion in apparent molecular volume of the solute relative to its native conformation.

Changes in molecular volume during refolding were derived from the elution volume measured at the peak apex. This parameter therefore represents the average hydrodynamic properties of the most abundant conformational species at a particular Gdn · HCl concentration. In comparison, the peakwidth of the solute zone includes contributions from a number of different conformational species involved in the

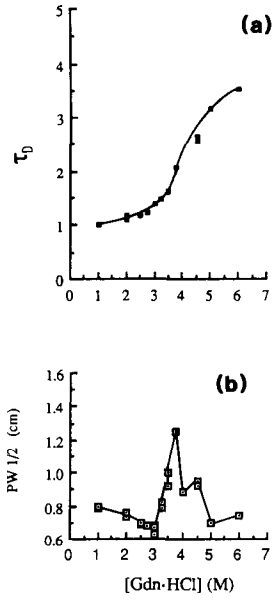

Fig. 3. (a) Equilibrium refolding of bGH as monitored by relative changes in K_d, *i.e.* τ_D with Gdn · HCl concentration. (b) The dependence of peak width at half peak height, $PW_{1/2}$ on Gdn · HCl concentration during the equilibrium refolding of bGH.

TABLE I

REFOLDING TRANSITIONS AND RELATIVE EXPANSION FACTORS OF PROTEINS AND FRAGMENTS USED IN THIS STUDY

	Transition (Gdn · HCl)	τ_D
bGH	3.8	3.5
pGH	3.5	3.6
hGH	4.7	4.1
oPrl	3.5	3.6
TD-bGH[a]	3.9	4.2
TD-pGH	3.3	4.3
TD-hGH	4.5	4.8
RA-bGH[b]	2–3	5.4
RA-pGH	2–3	5.5
RA-hGH	3.4	5.7

[a] TD: partial tryptic digest fragment.
[b] RA: reduced and alkylated.

eluting band, as well as the diffusional properties of these conformers. It is apparent from the data provided in Fig. 3b that there are significant changes in the experimental bandwidth as the Gdn · HCl concentration is increased. In particular, the presence of two distinct bandwidth maxima observed at *ca.* 3.8 and 4.5 *M* Gdn · HCl suggests the formation of at least two stable intermediates. Previous studies[15,17] on the equilibrium unfolding of bGH have demonstrated the reversible formation of a self-associated form of partially denatured bGH at *ca.* 3.7 *M* Gdn · HCl. The identity of the intermediate species, derived from the unfolding and refolding studies at 3.8 *M* Gdn · HCl was further investigated by monitoring the influence of protein concentration on the relative molecular volume of bGH at 3.8 *M* Gdn · HCl. The relative changes in K_d and peak width were assessed by chromatographing solutions of bGH ranging in protein concentration between 0.2 and 2 mg/ml. The results are shown in Fig. 4a and b, respectively, and demonstrate that while the K_d ratio exhibits an almost linear increase with protein concentration, the chromatographic peakwidth reaches a plateau at protein concentrations of *ca.* 1 mg/ml. The data indicate that at 0.3 mg/ml bGH exhibits a K_d value consistent with a monomeric structure. A value of $\tau_D = 4$ for the relative changes in molecular volume at a protein concentration of 2 mg/ml is consistent with the formation of a bGH species, under these partially-renaturing conditions, with a relative molecular volume corresponding to at least two monomer units. From the chromatographic data it is not immediately evident why there is a linear increase in the relative molecular volume rather than a discrete transition between the monomeric and dimeric species. However, the elution profiles obtained at 1 mg/ml exhibited solute peaks which tailed towards the lower molecular weight region. This observation suggests that the dilution of the bGH sample upon injection onto the Superose column results in interconversion of the monomer/dimer equilibrium during the separation, which then induces the formation of a small proportion of monomer. In the present study, the non-coincidence of the dependence of τ_D values and $PW_{1/2}$ on protein concentration suggests that these two parameters may be

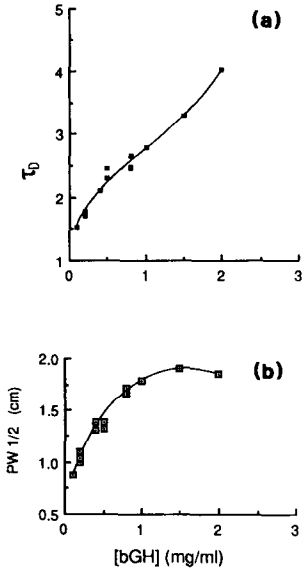

Fig. 4. The influence of bGH concentration on (a) relative molecular volume, τ_D, and (b) $PW_{1/2}$ at 3.7 *M* Gdn · HCl.

a measure of different aspects of this aggregation process. Thus, while the apparent molecular volume of the most abundant species gradually increased with increased protein concentration, the bandwidth reflects the extent of interconversion of the monomer–oligomer species.

It has previously been postulated by Brems *et al.*[18] that the self-association of bGH occurs through specific intermolecular interactions between the exposed hydrophobic face of the amphipathic helix encompassing amino acid residues 107–127. If the mechanisms of unfolding and refolding of GHs involve the formation and interaction of the same or similar structural intermediates, then porcine growth hormone (pGH), which exhibits 90% sequence homology with bGH and also contains an identical sequence between amino acid residues 107–127 except for a Gln/Leu substitution at position 121, should manifest very similar renaturation curves and concentration-dependent aggregation. In Fig. 5a and b, the relative molecular volume and $PW_{1/2}$ of pGH eluted on the Superose column are plotted against the Gdn · HCl concentration. The midpoint of renaturation occurred at 3.5 *M* Gdn · HCl which indicates that pGH is slightly less stable than bGH under these denaturing conditions. However, at 6 *M* Gdn · HCl, pGH increased in molecular volume by a factor of 3.6, which is very similar to the final extended molecular volume of bGH. The peak width data shown in Fig. 5b also shows a similar pattern to bGH with two maxima formed at 3.3 and 3.8 *M* Gdn · HCl, respectively. These results indicate that while at least two stable intermediates are formed during the renaturation of pGH, they also exhibit slightly diminished stability relative to the analogous bGH intermediates. This finding suggests that the intermolecular forces which are responsible for the existence of the transient conformers are also weaker in pGH than in bGH.

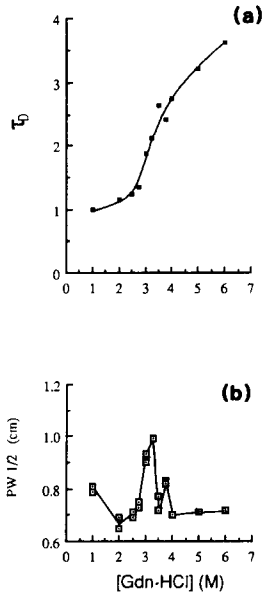

Fig. 5. (a) Equilibrium refolding of pGH as monitored by relative changes in molecular volume, τ_D, with Gdn · HCl concentrations. (b) The dependence of $PW_{1/2}$ on Gdn · HCl concentration during the equilibrium refolding of pGH.

hGH exhibits a 65% sequence homology with bGH and 74% homology with pGH, and displays marked differences in biological activity. The refolding behaviour of hGH in Gdn · HCl is shown in Fig. 6. Changes in relative molecular volume of hGH show the same sigmoidal shape as observed for both bGH and pGH. However, hGH was considerably more stable with a midpoint of refolding at 4.7 *M* Gdn · HCl compared with 3.8 and 3.5 *M* for bGH and pGH, respectively. Another difference in the behaviour of hGH was the tendency to form a small proportion of associated dimers at all Gdn · HCl concentrations, shown in Fig. 7 as a small peak eluting before the main monomeric species. Collection and reinjection of this dimer fraction resulted in identical elution profiles, which indicates that the dimeric form existed as a non-covalent aggregate in equilibrium with monomeric hGH. The renaturation curve of the hGH dimer is also shown in Fig. 6. While the relative molecular volume of the hGH dimer is larger in magnitude, the refolding behaviour appears to mimic that of the monomer. The persistence of the dimer even at 1 *M* Gdn · HCl also suggests that the molecular region involved in the intermolecular bonding may be located within relatively hydrophobic sections of the protein, which are known to be surface exposed in the native protein.

A further difference in the folding behaviour of hGH compared to bGH and pGH is illustrated by the pattern of bandwidth changes. Fig. 6b shows the influence of Gdn · HCl concentration on the monomer bandwidth which increased in a sigmoidal fashion with a midpoint value which corresponded to the transition value in the relative molecular volume. This result is in contrast to the existence of two bandwidth maxima observed for bGH and pGH which occurred in partially denaturing concentrations of Gdn · HCl. It is therefore apparent that hGH, compared to bGH or

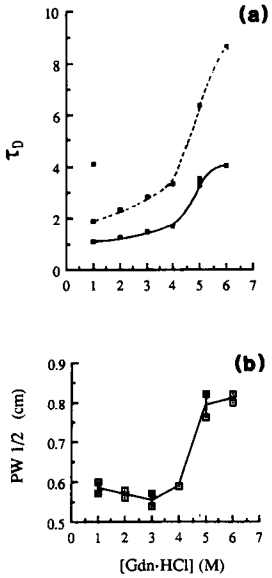

Fig. 6. (a) Equilibrium refolding of hGH as monitored by relative changes in molecular volume, τ_D. (b) The dependence of $PW_{1/2}$ on Gdn · HCl concentration during the equilibrium refolding of hGH.

pGH, follows a significantly different refolding pathway which reflects differences in the overall structural hierarchy within the molecule. These apparent differences between hGH and bGH or pGH in terms of the macroscopic secondary and tertiary structures may also be related to regional susceptibility of the three GH molecules to

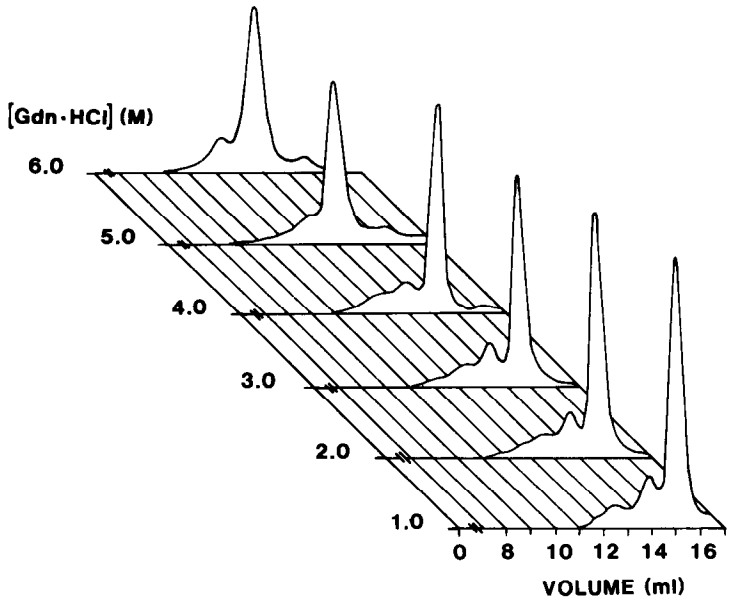

Fig. 7. HPSEC elution profiles of hGH as a function of Gdn · HCl concentration.

tryptic digestion[19]. For example, Fridman *et al.*[19] have found that bGH and pGH exhibited very similar surface structures while hGH exhibited a significantly different pattern of surface accessibility. In addition, recent studies by other workers[20] on the ultraviolet, resonant Raman and fluorescence spectroscopy of GHs, following acid-induced structural alterations, indicated the presence of a different environment around the single conserved tryptophan residue at position 86 in hGH compared to bGH and pGH. The results of the present study provide further confirmation of the overall differences in the structure and stability of the human growth hormone molecule compared to other structurally and functionally related proteins.

The hormone prolactin (Prl) is structurally related to the GHs but exhibits only lactogenic activity[12]. To further investigate the relationship between proteins of similar structure, the renaturation properties of ovine Prl (oPrl) were compared to the refolding behaviour of the three GHs. Fig. 8a shows the changes in relative molecular volume of oPrl as a function of Gdn · HCl concentration, and indicates a renaturation pattern similar to bGH and pGH rather than hGH. Thus, the midpoint of unfolding occurred at 3.5 *M* Gdn · HCl compared with 3.8, 3.5 and 4.7 *M* for bGH, pGH and hGH, respectively. The pattern of bandwidth changes during the unfolding of oPrl is shown in Fig. 8b. In contrast to bGH, pGH and also hGH, oPrl bandwidths increased between 5–6 *M* Gdn · HCl which correspond to the conditions in which the molecule was fully unfolded. Furthermore, there were no bandwidth maxima as observed for bGH and pGH which suggests that similar structural intermediates were not formed during the refolding process. The Prls exhibit approximately 20% sequence homology with hGH. While physicochemical studies have previously indicated[22] that Prls and GHs adopt similar conformations with approximately equivalent α-helical content, the present results indicate that there are significant differences in the intramolecular factors which control the refolding pathways of these proteins.

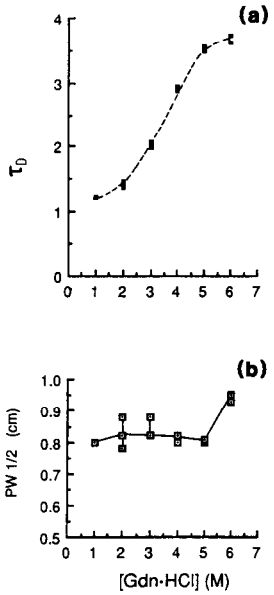

Fig. 8. Equilibrium refolding of oPrl in Gdn · HCl as monitored by (a) changes in τ_D and (b) changes in $PW_{1/2}$.

Chemically modified growth hormones

Limited tryptic digestion. Early reports have documented[21] that hGH, following partia· digestion with chrymotrypsin, retains significant somatotrophic activity. Subsequently there has been considerable effort in numerous laboratories directed towards identifying smaller fragments of GHs which retain potent biological activity (for recent review see ref. 22). To date, these approaches have not led to the successful molecular dissection of the somatotrophic and lactogenic activities within the sequential structure of hGH. However, it is known that significant biological activity can be maintained using two types of chemical modification of the GH molecule, namely (i) reduction followed by alkylation and (ii) limited proteolytic cleavage with trypsin[22,23]. The influence of these processes on the folding behaviour and relative stability of bGH, pGH and hGH was therefore investigated using analogous HPSEC methods to those described above.

Partial tryptic digestion of bGH produces a large fragment (TD-bGH) from which the segment encompassing amino acid residues 140–150 has been excised[19]. It is known from Chou-Fasman analyses[24] that this region of bGH is relatively unstructured with significant random coil features. This random coil region of bGH is also present in an analogous region in pGH[11]. Inspection of the X-ray crystal structure of pGH[11] reveals that this portion of the sequence is located within an extended random coil which connects helices 3 and 4. The resulting GH partial tryptic fragment therefore consists of two polypeptide chains corresponding to residues 1–139 and 151–191 which are connected by two disulphide bonds between Cys-53 and Cys-164 and between Cys-181 and Cys-189, respectively. The renaturation curve of TD-bGH which is shown in Fig. 9a shows a similar folding pattern to intact bGH with a refolding

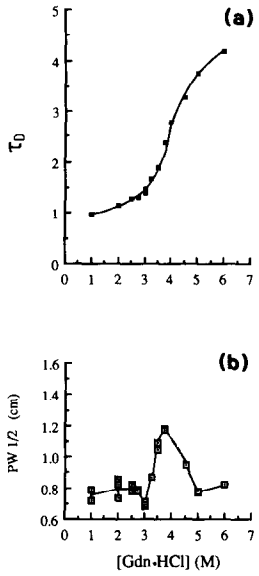

Fig. 9. Equilibrium refolding of TD-bGH in Gdn · HCl as monitored by (a) changes in τ_D and (b) changes in $PW_{1/2}$.

midpoint corresponding to 3.9 *M* Gdn · HCl reflecting a slightly more stable molecule. However at 6 *M* Gdn · HCl the apparent K_d of TD-bGH expanded by a factor of τ_D = 4.2 compared to τ_D = 3.5 for the intact bGH and is consistent with the properties of a more flexible molecule. The excision of residues 140–150 also resulted in a greater propensity of TD-bGH to polymerise which was evidenced by the presence of components of apparently larger molecular weight in the elution profiles. The excision site is adjacent to helix 3, *i.e.* residues 106–129, in the three-dimensional structure and this may result in the increased accessibility of the hydrophobic faces of this helix thereby facilitating aggregation. The dependence of bandwidth on Gdn · HCl concentration is shown in Fig. 9b and exhibits a maximum at 3.8 *M* Gdn · HCl which corresponds to the concentration value for the molecular volume transition.

The renaturation properties of the analogous tryptic derivative of pGH (TD-pGH) are shown in Fig. 10a and b. The midpoint of refolding occurred at 3.3 *M* Gdn · HCl which reflects a less stable structure than intact pGH. However, in 6 *M* Gdn · HCl, TD-pGH also existed as a more expanded open structure with a relative change in molecular volume of τ_D = 4.3 compared to τ_D = 3.6 for pGH. The pattern of bandwidth changes which was observed for bGH and pGH was also evident with TD-pGH. Thus, the presence of two peak maxima at 3 and 3.8 *M* Gdn · HCl in Fig. 10b, indicates the formation of two stable intermediates during the refolding of TD-pGH.

The partial tryptic fragment of hGH (TD-hGH) was also prepared and found to involve an analogous excision region between residues 135 and 145. The results of the Gdn · HCl induced renaturation are shown in Fig. 11a and b. The curves depicted in Fig. 11a correspond to different aggregates present at each concentration of

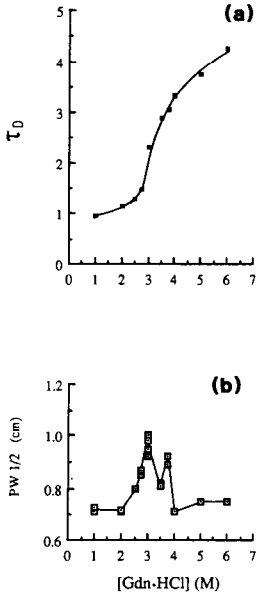

Fig. 10. Equilibrium refolding of TD-pGH in Gdn · HCl as monotired by (a) changes in τ_D and (b) changes in $PW_{1/2}$.

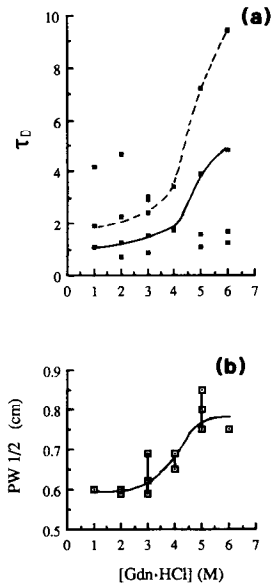

Fig. 11. Equilibrium refolding of TD-hGH in Gdn · HCl as monitored by (a) changes in τ_D and (b) changes in $PW_{1/2}$.

Gdn · HCl. Thus, the tendency of hGH to self-associate was not affected by tryptic cleavage. The bandwidth data also increased in a manner which reflects the changes in relative molecular volume of TD-hGH with Gdn · HCl concentration. These data also indicate that a more complex range of conformational and aggregational species occur with hGH derivatives in solution under denaturing conditions, compared to the bGH or pGH derivatives. Overall, therefore, the loss of a small fragment of the hGH protein from within a random coil section of the molecule does not appear to significantly alter the stability or the folding behaviour, but only influences the extent by which the hGH molecule can unfold in fully denaturing conditions. These conclusions are consistent with the results of other studies which have demostrated that this tryptic fragment of hGH maintains full biological activity[22]. This observation suggests that the region encompassing residues 135–150 is not important in the maintenance of structure and/or the biological function.

Reduction and alkylation. The role of the disulphide bonds in the maintenance of conformation and structural stability of growth hormones was investigated by studying the renaturation behaviour of reduced and carboxymethylated derivatives of bGH, pGH and hGH. This process results in the disruption of the two disulphide bonds between Cys-53–Cys-164 and Cys-181–Cys-189 and also introduces a negative charge on each cysteine through a carboxymethylation reaction.

The chromatographic profiles obtained for the elution of reduced and alkylated bGH (RA-bGH) in different concentrations of Gdn · HCl are shown in Fig. 12. Between 0–4 *M* Gdn · HCl, complex multicomponent elution profiles were obtained, indicative of the presence of various aggregated forms of RA-bGH. While the elution volume for each form was constant at each concentration of Gdn · HCl, the relative

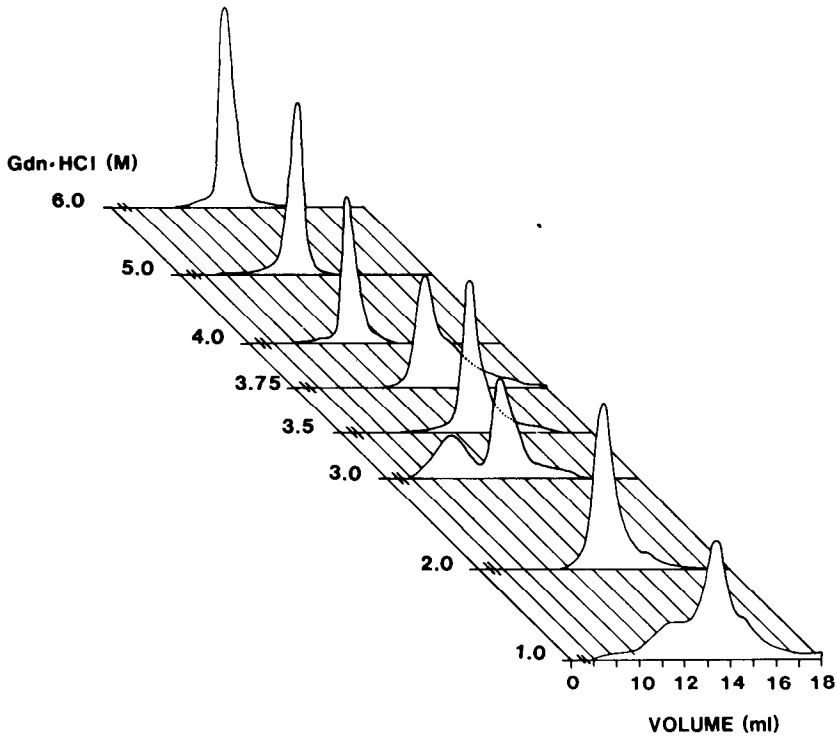

Fig. 12. HPSEC elution profiles of RA-bGH as a function of Gdn · HCl concentrations.

proportion of each aggregate varied with consecutive injections in a protein concentration dependent manner. Under the conditions of these experiments where the protein is refolding from 6 *M* Gdn · HCl, the variation in experimental peak heights corresponding to each aggregate/conformer suggests that they may represent kinetic intermediates along a relatively slow refolding or aggregation pathway. The renaturation curve for RA-bGH is shown in Fig. 13 and shows the refolding pathway of a monomer, dimer, trimer and a decamer. Between 1–3 *M* Gdn · HCl, the predominant form corresponded to an apparent dimer with a relative molecular volume of τ_D = 1.8–2.2 (compared to monomer). Between 4–6 *M* Gdn · HCl, a reproducible single peak was observed with a relative molecular volume of τ_D = 5.4 units. When compared to values of τ_D = 3.5 and τ_D = 4.2 for bGH and TD-bGH, respectively, it thus appears that the monomer is the major species for carboxymethylated bGH under fully denaturing conditions. Similar results were also obtained for the reduced and alkylated derivative of pGH (RA-pGH). While it is not possible from the experimental data to fully delineate the renaturation curve for the monomeric species (because of the presence of higher aggregates), the disruption of the intermolecular forces involved in the formation of the dimer and the transition point for the monomer refolding coincided in the range between 2–3 *M* Gdn · HCl. This value is significantly lower than the midpoint of refolding for both bGH and TD-bGH. Previous studies on the equilibrium unfolding of bGH[25] have indicated that the large loop formed between

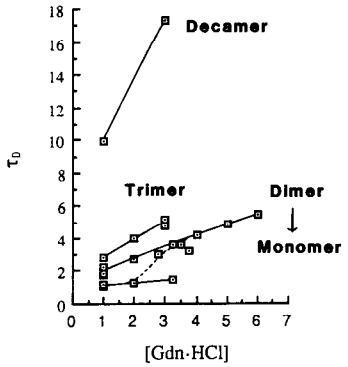

Fig. 13. Equilibrium refolding of RA-bGH as monitored by changes in τ_D.

Cys-53 and Cys-164 plays a major role in secondary structure stabilisation, while the small section between Cys-181 and Cys-189 does not impart any significant stability to the native structure. The results of the present study indicate that reduction and alkylation of bGH and pGH leads to a diminished stability of the tertiary structure presumably due to the exposure of the hydrophobic interior of the molecule which promotes dimerisation through intermolecular interaction between the exposed amphipathic helices.

The chromatographic profiles of RA-hGH are shown in Fig. 14. In 6 *M* Gdn · HCl the relative molecular volume of RA-hGH corresponded to $\tau_D = 5.7$ which demonstrates a significantly larger and more flexible molecule than intact hGH. In contrast to the results observed for RA-bGH and RA-pGH, under non-denaturing

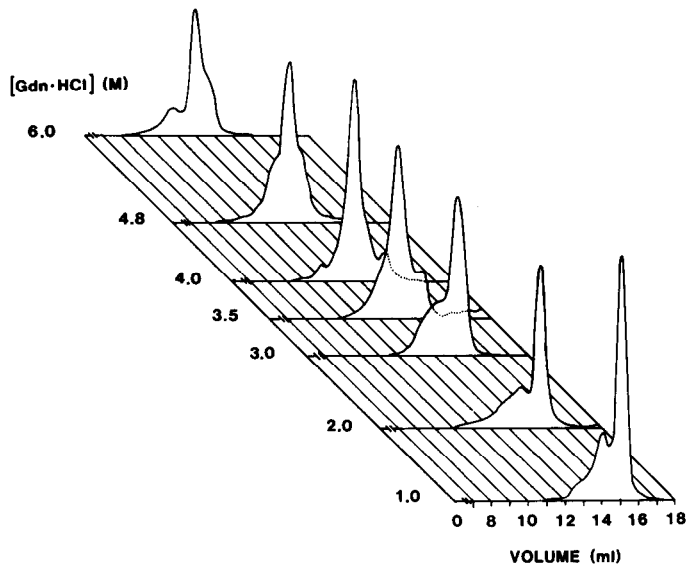

Fig. 14. HPSEC elution profiles of RA-hGH as a function of Gdn · HCl concentrations.

conditions RA-hGH chromatographed predominantly as a monomer, with only a very small proportion of a dimeric form present. The stability of the RA-hGH monomer was significantly decreased relative to hGH, with a midpoint of refolding of 3.4 *M* Gdn · HCl compared to 4.7 *M* for unmodified hGH. This result also provides further insight into the origin of the differences in the folding stability of bGH and pGH compared to hGH, and can be correlated with the influence of reductive alkylation procedures on the biological activity of GHs. For example, reduction and carboxymethylation of hGH causes a loss of growth promoting activity but not lactogenic activity[23]. In contrast, RA-pGH is totally inactive. The disulphide bridge formed between Cys-53 and Cys-164 results in the covalent linkage of a random coil segment and helix 4. Disruption of this disulphide bond alters the topographical arrangement of the amino acid residues within the microenvironment of this region, which may in turn lead to significant effects on the growth promoting activity. While the present study investigates the propensity of the GHs to refold, the unfolding pathway may not necessarily involve the same intermediate steps. Studies into the relationship between the unfolding and refolding of the GHs and the specific role of each helical segment in the aggregation process will be presented in a future paper.

CONCLUSION

The results of the present study further demonstrate the utility of HPSEC in the characterization of protein folding and stability. In particular, the experimental differences observed between the stability and refolding behaviour of hGH and the non-primate molecules bGH, pGH and oPrl confirms other observations that the human molecule is unique in terms of its three-dimensional structure and stability. Previous studies from our laboratories have demonstrated the potential of interactive modes of chromatography, such as reversed-phase[26] and ion-exchange[27], to probe the surface topography and characterise the relative stability of peptides and proteins. The present study further documents HPSEC as an additional dimension in the chromatographic repertoire which can be employed in combination with other techniques for the investigation and characterization of structure–retention-function relationships of medically important proteins.

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