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A relationship between the starting secondary structure of recombinant porcine growth hormone solubilised from inclusion bodies and the yield of native (monomeric) protein after in vitro refolding

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Recombinant porcine growth hormone (rPGH) was solubilised from inclusion bodies (IB's) using either 6 M guanidinium hydrochloride (GnHCl), 7.5 M urea or by a novel method using a cationic surfactant, cetyltrimethylammonium chloride (CTAC). Circular dichroism (CD) analysis of the secondary (2°) structure of the urea- and GnHCl-solubilised rPGH showed the absence of α-helical content with the majority of the molecule existing in a 'random coil' structure. In contrast, the CTAC-solubilised rPGH displayed significant starting 2° structure (10–15% α helix; 30–40% β structure). The three rPGH preparations were refolded in vitro against weak urea, GnHCl or aqueous buffers, resulting in an average refolding efficiency of 50% native (monomeric) rPGH for CTAC solubilised IB's and only 20% for urea or GnHCl solubilised IB's. We conclude that the method of solubilisation of IB's and the resultant difference in the starting 2° structure of rPGH, particularly α-helical content, is a major in vitro factor that apparently predetermines the aggregation/refolding behaviour rPGH irrespective of refolding environment.

In vitro refolding; Secondary structure; Cationic surfactant; Growth hormone; Inclusion body

1. INTRODUCTION

In common with the high-level expression of many eukaryotic proteins in bacteria, expression of animal growth hormones in *E. coli* results in their deposition within large proteinaceous aggregates or inclusion bodies [1–5]. The formation of these IB's is often considered undesirable since the deposited protein can only be solubilised using strong denaturants such as 6 M GnHCl, or 7.5 M urea or under other harsh conditions [6–9]. The use of high concentrations of urea and/or GnHCl results in the loss of secondary structure of solubilised proteins with the protein molecule existing mostly in the so called 'random coil' formation [10].

The denaturation of recombinant proteins during solubilisation from IB's necessitates that they be 'refolded', that is, renatured in vitro to regain native confirmation. For intramolecular disulphide linked proteins such as rPGH, the in vitro formation of undesirable high molecular-weight aggregates due to aberrant intermolecular disulphide bonding is a major problem limiting recovery of native (i.e. monomeric 22K) protein. We have recently reported a novel method for solubilising recombinant proteins from IB's [11] using a cationic surfactant, cetyltrimethylammonium chloride (CTAC). We show here that rPGH solubilised using CTAC displays considerable differences in secondary structure, particularly α-helical content, in comparison with urea/GnHCl solubilised rPGH. The relationship between different starting secondary structure and subsequent in vitro refolding yield and efficiency of formation of native (monomeric) over aggregated (denatured) rPGH was examined for three different refolding environments and the results related to the 'framework' model of protein folding [12].

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2. MATERIALS AND METHODS

2.1. *Solubilisation and refolding of rPGH*

Methionyl rPGH was expressed in *E. coli* and IB's purified as described previously [11,13]. The IB's from fermentation batch F135 (dry weight 90 mg/ml) were solubilised in 25 mM ethanolamine-HCl pH 10.0 containing 1% (v/v) 2-mercaptoethanol and either 7.5 M urea, 6.0 M GnHCl or 5% (w/v) CTAC for 1–2 h at ambient temperature or 55°C as described previously [11,13]. Approximately 50 mg dry weight of IB's was solubilised in 3 ml of each buffer and 1 ml aliquots of each of the solubilised rPGH preparations were subsequently refolded by dialysis, at a concentration of 2–3 mg/ml against 25 mM ethanolamine-HCl pH 10.0 with or without 3 M urea or 1 M GnHCl. Refolding was for 48–72 h at 4°C with shaking and aeration and a total of 9 different solubilisation/refolding combinations resulted.

2.2. *HPLC analysis*

Reversed-phase HPLC (RP-HPLC) analyses to quantitate the amount of oxidised and monomeric rPGH as a % of the total refolded protein (monomeric and aggregated fractions) were performed and validated with purified oxidised monomeric, reduced, and 'aggregated' rPGH standards as described previously [11,13].

2.3. *Preparation of IB material for CD analyses*

For CD analyses, IB's were further purified by sucrose-gradient centrifugation. Briefly, the IBs were resuspended in 10 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA pH 8.0 and layered onto a stepwise gradient consisting of equal volumes of 67%, 53% and 40% (w/v) sucrose in 1 mM Tris-HCl, 1 mM EDTA, pH 8.0. The final gradient was established by centrifugation at 108,000×*g* for 2 h. The IB's appeared as a white band at the 53–67% sucrose interface and were collected with a Pasteur pipette, and washed thrice with MilliQ water. The final protein pellet, equivalent to approximately 45 mg dry weight was resuspended in 4.5 ml of solubilisation buffer containing 6.4 M GnHCl, 8.0 M urea or 5% (w/v) CTAC in 50 mM glycine, 0.25 M 2-mercaptoethanol pH 10. Solubilisation was conducted for 1 h at 55°C. After solubilisation, the free thiols on the protein were blocked by the addition of 0.5 ml of a 2 M solution of cysteine in 0.6 M HCl and the mixture further incubated for 30 min at 55°C. The solubilised samples were then exchanged twice with buffers containing 6.4 M GnHCl, 8.0 M urea or 5% (w/v) CTAC in 50 mM glycine; pH 9.1 using Centricon-10 concentrators (Amicon). The solubilised samples were filtered through a 0.22 μm filter and then centrifuged at 13,000×*g* for 5 min prior to use.

2.4. *Protein estimations*

Samples were precipitated with ethanol (90% (v/v) final concentration), vortexed, incubated at −20°C for 30 min, centrifuged at 13,000×*g* for 15 min, resuspended in 0.225 M NaOH and protein content estimated by the Lowry method [14].

2.5. Circular dichroism (CD) measurements

CD analyses were conducted as described previously [11]. CD measurements were conducted in the presence of the solubilisation reagent used, that is, 6.4 M Gn-HCl, 8.0 M urea or 5% (w/v) CTAC in 50 mM glycine buffer pH 9.1.

3. RESULTS AND DISCUSSION

3.1. *Secondary structure of solubilised rPGH preparations*

The peptide absorption spectra (λ < 240 nm) of reduced rPGH preparations solubilised from IB's were examined between 200–250 nm in order to estimate the secondary structure of urea-, GnHCl- and CTAC-solubilised proteins. Typical CD spectra are shown in Fig. 1. Notable differences were apparent between the CD spectrum of CTAC-solubilised rPGH and the spectral curves for urea/GnHCl-solubilised protein. The CTAC-solubilised proteins were calculated to comprise approximately 10–15% α helix; 10–15% β sheet; 20–25% β turn and 40–50% 'random-coil' structure. In contrast, the urea/GnHCl-derived rPGH displayed no α-helical structure, apparently comprising 40–50% β turns and 40–50% random coil structure. While the large components of apparently unordered 'random coil' structures in these proteins mean that the values for ordered structural components (α-helical and β structures) should be regarded as approximate, the differences in values (particularly α helix) clearly reflect the observed differences in the respective shapes of the CD spectral curves; notably at the negative n-π* band near 220 nm which is characteristic of α-helical content (cf. Fig. 1, curve c). The values for β turns/sheet and 'random coil' structure assignments are generally regarded as less reliable than

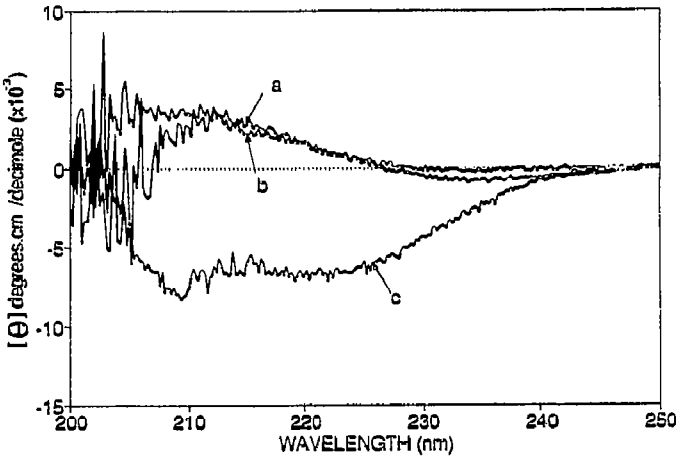

Fig. 1. CD spectra of rPGH solubilised from IB's using in part (a) 8.0 M urea/0.25 M 2-mercaptoethanol; (b) 6 M GnHCl/0.25 M 2-mercaptoethanol and (c) 5% (w/v) CTAC and 0.25 M 2-mercaptoethanol. The θ values are the calculated measures of the mean residue ellipticities. CD spectral analysis was performed as described previously [11].

for α helix, within the accepted limits of CD analysis of denatured proteins (17–19).

3.2. *Refolding of urea, GnHCl- and CTAC-solubilised rPGH preparations*

Recombinant proteins solubilised from IB's using chaotrophic agents in a 'strongly' denaturing environment (7.5 M urea or 6 M GnHCl) are commonly renatured in vitro in 'weakly' denaturing environments using 0.5–2 M GnHCl or 1–4 M urea [7–9,15]. To examine the relative contribution of different methods of solubilisation and therefore the starting 2° structure, and the in vitro refolding environment itself on yield of native (i.e. monomeric 21.5K) rPGH, we used three simple refolding environments; 3 M urea, 1 M GnHCl or a weak aqueous buffer for each of the three solubilised rPGH preparations. A total of 9 different solubilisation-refolding combinations were therefore examined. The proportion of native (monomeric) rPGH after refolding was estimated by RP-HPLC using a method we have previously validated and used to separate monomeric 21.5K rPGH from polydisperse high mol. wt. rPGH or other contaminants [11,13]. A typical RP-HPLC profile of refolded rPGH is shown in Fig. 2, with differences in retention time of between 1–1.5 min for monomeric (native) and 'aggregated' rPGH or other *E. coli* contaminants.

The results of the nine solubilisation–refolding combinations examined are shown in Table I as the % yield of monomeric rPGH in each case, as well as an average % refolding efficiency for either CTAC, urea or GnHCl solubilised IB's. For a given solubilisation–refolding combination, the % yield of monomeric rPGH using CTAC-solubilised IB's was clearly between 2- to 4-fold greater than that with urea- or GnHCl-solubilised IB's,

irrespective of the in vitro refolding environment used (Table I; column 3). Moreover, the average efficiency of formation of native rPGH was respectively 50% for CTAC-solubilised against 20% for urea- or GnHCl-solubilised IB's, suggesting that selection of the method of solubilisation and the resulting differences in secondary structure were more important for influencing the yield of native protein than the in vitro refolding environment.

4. CONCLUSIONS

We have used in this study a novel method of solubilising rPGH from IB's with significant retention of 2° structure, notably α-helical content. This has allowed for the first time an experimental determination of whether the starting 2° structure of a protein (rPGH) solubilised using surfactant, urea or GnHCl in any way predisposes the protein to a particular in vitro refolding pathway, or whether the in vitro refolding environment used is the major influence on the proportion of native rPGH recovered. Our results clearly show that rPGH from IB's solubilised using the cationic surfactant CTAC was substantially less prone to in vitro aggregation, resulting in higher average recovery of native structure irrespective of the three refolding environments used. This effect is unlikely to be due to CTAC offering any 'nonspecific' protection against aggregation as any residual surfactant bound to protein is removed by a cation exchange step prior to commencing in vitro refolding.

Table I

The effect of various solubilisation and in-vitro refolding environments on the yield of monomeric (native) rPGH

Method of solubilisation	Method of refolding[1]	% monomeric rPGH[2]	Average % refolding efficiency[3]
1. 5% (w/v) CTAC	aqueous buffer	20	
	3 M urea	33	50
	1 M GnHCl	20	
2. 7.5 M Urea	aqueous buffer	10	
	3 M urea	15	22
	1 M GnHCl	10	
3. 6 M GnHCl	aqueous buffer	6	
	3 M urea	15	20
	1 M GnHCl	8	

[1] Refolding was performed in 25 mM ethanolamine-HCl buffer pH 10.0 with and without 3 M urea or 1 M GnHCl at a rPGH concentration of approximately 2 mg/ml for 48–72 h at 4°C with aeration.

[2] % monomeric rPGH was estimated by RP-HPLC as validated and described previously [11,13].

[3] Calculated as the average of the % monomeric rPGH yields shown in column 3, that is, 25%, 11% and 10% for respectively the CTAC-, urea- and GnHCl-solubilised rPGH divided by the percentage of reduced rPGH at the start of in vitro refolding as previously determined for the IB's used [11].

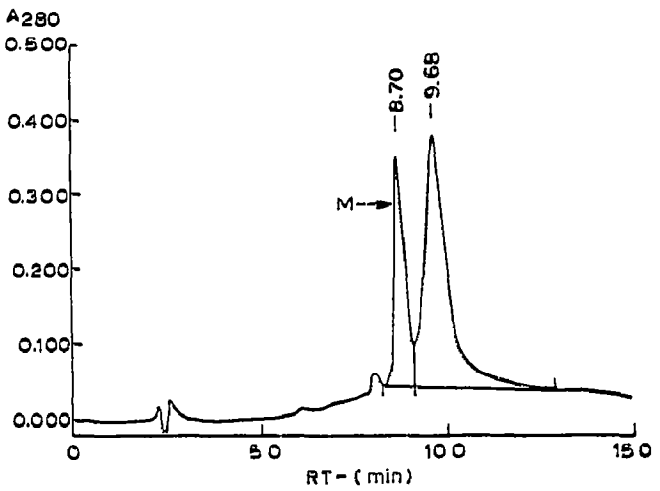

Fig. 2. Example of RP-HPLC analysis of in vitro refolded rPGH solubilised from IB's using CTAC. The monomeric (M) rPGH peak is clearly resolvable from polydisperse 'aggregated' rPGH and *E. coli* contaminants, as validated and described previously [11,13] using SDS-PAGE and standard preparations of monomeric rPGH.

We have previously established using CD analysis that correctly refolded rPGH comprises approximately 35–40% α helix [11]. In the present study, reduced, CTAC-solubilised, that is, 'unfolded' rPGH still comprised approximately 10–15% α helix. It would appear that the notable differences in starting secondary structure between CTAC-urea- and GnHCl-solubilised rPGH do predispose the in vitro refolding behaviour of rPGH although a direct casual relationship with yield of native, monomeric protein remains to be established. However, these results are consistent with the 'framework' model of protein folding [12] which postulates that the presence of localised secondary structures plays a central role in determining the folding pathway, as well as a recent report [16] demonstrating the native-like structure of refolding intermediates. This suggests that the structures of refolding intermediates and the protein folding pathway reflect the stability of secondary structural units and assemblies that would normally be found in the native protein. In the case of surfactant-solubilised rPGH, starting in vitro refolding with an apparent 'intermediate' displaying definite 2° structural components of the native state, is clearly beneficial. On a more practical level, the use of CTAC to solubilise other recombinant proteins from IB's may represent an approach offering similar advantageous in vitro refolding behaviour to that reported here for rPGH. Alternatively, the conditions for solubilisation of insoluble recombinant proteins, particularly the GnHCl or urea concentration used, could be carefully selected to give an acceptable level of solubilisation together with, if possible, retention of some protein 2° structure prior to commencing in vitro refolding.

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