Characterisation of Intermediate(s) in the Folding Pathway of Porcine Growth Hormone

by

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THESIS SUMMARY

The equilibrium denaturation of recombinant porcine growth hormone (pGH) demonstrated that pGH does not follow a simple two-state folding mechanism, but is consistent with the framework model of folding (Bastiras and Wallace, 1992). Stable intermediates observed were similar to other non-human growth hormones and characterised as compact and largely α-helical, yet lacked native-like tertiary structure; characteristics of the molten globule state. The detection of intermediate states along a protein's folding pathway, is crucial to understanding the principles of protein folding. To further understand the folding mechanism of pGH, this thesis describes the acid-induced denaturation and folding kinetics of recombinant pGH, 3 site-directed mutants (pGH analogues) and recombinant rat growth hormone (rGH).

The acid–induced denaturation of pGH, in the absence and presence of 4 M urea, was monitored by a variety of physicochemical techniques. Changes in tertiary structure were followed by (i) UV absorption spectroscopy (including second derivative analysis), (ii) intrinsic tryptophan fluorescence and (iii) near-UV circular dichroism. Secondary structural changes were followed by far-UV circular dichroism. Changes in the hydrodynamic radius self-association were followed by size-exclusion chromatography and analytical ultracentrifugation. The hydrophobic dye 1-anilinonapthalene-8-sulfonate (ANS) was also used to probe conformational changes upon acidification. Acidification alone (pH 8.0 to pH 2.0) of pGH resulted in changes in intrinsic fluorescence, UV absorbance, and near-UV CD, with transitions centred at pH 4.1. At pH 2.0, a red shift in the fluorescence emission maximum of approximately 3 nm and a 15% loss of the far-UV CD signal at 222 nm implied that the protein did not become extensively unfolded. Acidification in the presence

of 4 M urea resulted in similar pH-dependent transitions, however, these occurred at a more alkaline pH. At pH 2.0 + 4 M urea, an 8 nm red shift in the fluorescence emission maximum suggested that unfolding was greater than in the absence of urea. Sedimentation equilibrium experiments in the analytical ultracentrifuge showed that native pGH and partially unfolded intermediates reversibly self-associate. The model of association of the partially unfolded intermediates in the absence and presence of 4 M urea was different. These results demonstrate that acidification of pGH in the absence or presence of 4 M urea induced the formation of molten globule-like states with measurable differences in conformation.

Previous equilibrium denaturation studies of recombinant bovine growth hormone (bGH) showed that a peptide fragment corresponding to helix 3 of bGH could inhibit molecular association (Brems *et al.*, 1986). The amino acid sequence encompassing the third helix of pGH is identical in sequence to helix 3 of bGH except for a leucine to glutamine substitution at position 121. The fragment composed of residues 96-133 from native pGH was isolated by partial tryptic digestion and subsequently purified by reverse phase HPLC. The presence of this pGH fragment during the acid-induced denaturation of pGH was found to reduce the self-association of partially unfolded intermediates at pH 2.0 in the presence of 4 M urea, but had no significant effect on self-association at pH 2.0 in the absence of 4 M urea.

The acid-induced denaturation of the 3 pGH analogues and rGH was characterised. In terms of acid-mediated partial unfolding, the four proteins in this study, similar to wild-type pGH, possess folding intermediates with classic characteristics of the molten globule. Experimental evidence suggested that the mutations invoked subtle differences in

conformation between the intermediates, which were dependent on the solvent system employed.

The folding kinetics of pGH, the 3 pGH analogues and rGH were studied using stopped-flow fluorescence spectroscopy. Specifically, the intrinsic fluorescence of the single internalised tryptophan at position 86 within helix 2 (Trp86) was used to monitor the folding mechanism. Kinetic experiments employing the single-jump method were analysed. Concentration jumps from 5 M to 1.5 M Gdn–HCl and 0 M to 4 M Gdn–HCl for refolding and unfolding, respectively, were employed. For wild-type pGH refolding, two kinetic phases, accounting for the total expected amplitude were resolved. Unfolding experiments resulted in one kinetic phase representative of approximately 60% of the expected amplitude change. An un-observed phase, more commonly termed 'burst phase' accounting for the remaining amplitude, occurred within the dead-time of mixing (4 ms) of the stopped-flow instrument. The effect of protein and Gdn–HCl denaturant concentration was also examined. The folding kinetics of the pGH analogues and rGH exhibited similarities in the number of kinetic phases resolved for both refolding and unfolding, however, the rate constants differed. These results are compared to those for wild-type pGH and are interpreted in terms of the stability of folding intermediates.