ABSTRACT

BACKGROUND TO CHAPTER 1: The structural consequences of polypeptide backbone reversal ('retro' modification) remain largely unexplored, in particular, for the retro forms of globular all-beta sheet proteins. To examine whether the backbone-reversed forms of model all-beta sheet proteins can fold and adopt secondary and tertiary structure, we created and examined three backbone-reversed proteins. The results were expected to shed some light on the role of backbone polarity in folding phenomena.

(i) We created the recombinant retro form of a 110 residues-long polypeptide, an α-crystallin-like small heat shock protein (sHSP), HSP12.6, from C.elegans. Following intracellular overexpression in fusion with a histidine-affinity tag in Escherichia coli, purification under denaturing conditions, and removal of denaturant through dialysis, retro-HSP12.6 was found to fold to a soluble state. The folded protein was examined using fluorescence and CD spectroscopy, gel filtration chromatography, differential scanning calorimetry and electron microscopy, and confirmed to have adopted secondary structure and assembled into a 12-mer. Interestingly, like its parent polypeptide, retro-HSP12.6 did not aggregate upon heating; rather, heating resulted in structural consolidation, and heated and cooled samples appear to retain some of this structural consolidation to yield a protein with an even more well-defined CD spectrum. Some preliminary crystals were obtained.

(ii) We created and examined the recombinant retro form of a 94 residues-long protein, the GroES co-chaperone of Escherichia coli. Following intracellular overexpression in E. coli in fusion with a 6XHis affinity tag, purification under denaturing conditions, and removal of denaturant through dialysis, retro-GroES was found to adopt a state that was soluble up to a concentration of approximately 3.5 mg/ml in pH 8.0 aqueous buffer. The protein was confirmed to have adopted secondary structure through circular dichroism and FT-IR spectroscopy. It was also confirmed to have assembled into a multimer with the apparent hydrodynamic volume of a tetramer, using gel filtration chromatography. The

multimer was dissociable by urea. Interestingly, the protein displayed reversible consolidation of structure upon heating to temperatures as high as 90 °C, rather than thermal structural melting, with no attendant aggregation. Heated-cooled samples and unheated samples displayed identical secondary structural contents; however, heated-cooled protein showed a smaller hydrodynamic volume. Preliminary crystals were obtained.

(iii) We then examined yet another backbone-reversed all-beta sheet protein. The backbone-reversed, or 'retro', form of a model all-beta sheet protein, Escherichia coli CspA, was produced from a synthetic gene in E.coli in fusion with an N-terminal affinity tag. Following purification under denaturing conditions and dialysis-based removal of urea, the protein was found to fold into a soluble, poorly-structured multimer. Upon concentration, this state readily transformed into amyloid fibers. Congo red-binding amorphous forms were also observed. Since a beta sheet-forming sequence is expected to retain high beta sheet-forming propensity even after backbone reversal, and given the fact that folding of retro-CspA occurs only to a poorly-structured form, we conclude that the increase effected in protein concentration may be responsible for the formation of intermolecular beta-sheets, facilitating the bleeding away of the protein's conformational equilibrium into aggregates.

BACKGROUND TO CHAPTER 2: The $(\beta/\alpha)_8$-barrel (TIM barrel) domain consists of eight topologically equivalent supersecondary structural motifs known as β/α units. Each unit consists of a single β-strand, an α-helix, and two loops. Evidence has accumulated in recent years to indicate that the $(\beta/\alpha)_8$-barrel motif may not be a single autonomously folding domain as earlier assumed. Segments of some $(\beta/\alpha)_8$-barrels appear to fold autonomously. However, the extent to which this is true of various $(\beta/\alpha)_8$-barrel domains remains to be explored. The results were expected to throw some light on the degree of autonomy of β/α sub-structures.

We scrambled (reshuffled) the native order of β/α units [1-2-3-4-5-6-7-8] comprising the polypeptide chain of a model $(\beta/\alpha)_8$-barrel from S.cerevisiae, triosephate isomerase (TIM). Total scrambling was effected to examine

whether folding can still occur to yield β/α structures in spite of a global 'destruction' of native hydrophobic and hydrogen bonding interactions between β/α units, as long as scope is maintained for the occurrence of native interactions within individual units. Our results demonstrate that scrambled full-barrel forms [2-4-6-8-1-3-5-7 and 1-3-5-7-2-4-6-8], as well as half-barrel [2-4-6-8] and quarter-barrel [1-3] forms of TIM fold into β/α structures that sustain tertiary and quaternary structural interactions. In particular, one variant [2-4-6-8-1-3-5-7] was found to fold and form a stable dimer with native-like structural content and other characteristics. Our results demonstrate that $(\beta/\alpha)_8$-barrels can tolerate profound alterations of (a) strand-strand interactions responsible for the creation of the β-barrel, as well as (b) the geometry of presentation of nonpolar sidechains into the hydrophobic core of the β-barrel by individual β-strands. These findings lend support to our recent proposal [Maiti et al., (2002). IUBMB Life 54, 213-221] that a hierarchy of interactions probably regulates structure formation and stability in $(\beta/\alpha)_8$-barrels, with folding proceeds successively through (i) the tentative formation of individual β/α units which associate through 'near-neighbor' diffusion-collision interactions into (ii) curved assemblies of multiple β/α units through sequence-independent hydrogen bonding of strands of neighboring units, leading finally to (iii) the association of curved (quarter/half-barrel) assemblies around a common hydrophobic core through packing interactions that remain plastic and amenable to change.

BACKGROUND TO CHAPTER 3: Some sequence-recombined constructs showed a tendency to aggregate at high concentrations. We were interested in examining whether such aggregates constitute entirely disordered coagulates of unstructured polypeptides, or ordered assemblies. We were also interested in examining whether aggregation involves homomolecular recognition, or heteromolecular recognition. These results were expected to shed light on the nature and quantity of molecular structure in aggregates of proteins with profound modifications of sequences carrying folding information.

Using two of the backbone-reversed constructs, retro-CspA and retro-HSP12.6, we applied microscopic methods to addressing the issue of whether or not the two

proteins influence each other's aggregation when they are caused to aggregate within the same tube through increase of protein concentration. Thus, aggregates of retro-CspA and retro-HSP12.6 were examined both singly and in mixtures, in unlabelled and labelled (fluorescent) forms. EM studies showed that the two proteins form amyloid-like structures of very different morphologies. However, coaggregates display morphologies that are different enough to indicate that the two proteins interfere with each other's amyloid-forming tendencies when they are aggregated in the same tube. Likewise, a molecular paint-mixing strategy using FITC-labeled retro-CspA and TRITC-labeled retro-HSP12.6 was used together with fluorescence microscopy to show that the fluorescence emission of co-aggregates owes to mixing of the emissions of the two proteins at the molecular level. This result - which indicated that retro-CspA and retro-HSP12.6 exist 'cheek-by-jowl' within aggregates when they are co-aggregated - was further confirmed using confocal fluorescence microscopy and spectral imaging. We were able to demonstrate by designing and adopting requisite optical bench specifications (i.e., laser excitation wavelengths, filter characteristics, and mirror, beam-splitters and plate settings) that the smallest confocal sections of co-aggregates, which are reckoned to contain <1000 molecules in the x-y plane, contain both FITC- and TRITC-labeled species. This was established: (a) by demonstrating that imaged aggregate particles show both FITC as well TRITC emission spectra, and (ii) by demonstrating that colocalization of FITC-labeled and TRITC-labeled species was applicable within all sections of aggregates in a field of co-aggregates.