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A Reversible Unfolding Reaction of Swine Pepsin; Implications for Pepsinogen's Folding Mechanism

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Summary Above pH 6, swine pepsin undergoes a conformational change to a neutral form which has 80% of the secondary structure of the native protein. In contrast to native pepsin, this form of the enzyme can be reversibly unfolded by urea in a rapid, cooperative reaction. Since all of pepsin's sequence is present in its precursor pepsinogen, it is likely that this neutral structure is present in one or more of the transient intermediates previously detected in the reversible unfolding reaction of the zymogen. The mechanism of this rapid reaction may resemble early steps in protein folding.

Introduction Extensive studies have shown that the denaturation of swine pepsin is irreversible, even when the enzyme's activity is inhibited to prevent autolysis. In contrast, unfolding of its precursor pepsinogen can be fully reversed, under the correct experimental conditions (1). Refolding of pepsin has always been attempted under conditions where it shows activity, below pH 6, whereas studies on pepsinogen are carried out above this value, since at acid pH, pepsinogen autoactivates to pepsin. In view of the fact that attempts to refold pepsinogen below pH 6 have also proved unsuccessful (2), it was essential to compare the behaviour of these two proteins under conditions where at least one of them is able to fully refold.

Materials and Methods Swine Pepsinogen (lot no. 34F-0020) was obtained from Sigma and used without further purification. Pepstatin A was from Peninsula Laboratories and the activation peptide containing residues 1-16 of pepsinogen's sequence was synthesized by Biosearch. "Ultra Pure" urea was from Mann. Diazoacetylglycine ethyl ester inactivated pepsin (DAG-pepsin) was prepared from pepsinogen as described before (1). Protein concentrations were measured using molar extinction coefficients of 51,000 at 278nm. Stock solutions (1mg/ml) were made up in buffers, pH 4.5 or 7.5, with or without 10M urea, and small aliquots (50uL) added to 3.0mL of buffers, (0.1M solutions of sodium phosphate, monobasic, adjusted to the required pH with sodium hydroxide), at the required concentration of urea.

Circular Dichroism (CD) spectra were measured in a Jasco J500-C spectropolarimeter, with a DP-500N data processor. Solutions were held in 1cm pathlength quartz cuvettes in a cell holder thermostated at 25° C with a Lauda circulating water bath. Spectra were routinely scanned four times and averaged. The usual precautions with regard to slit width and absorbance were observed.

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Calculations were performed on a DEC-10 computer, using the MLAB modelling system (3).

Results Pepsin undergoes a substantial change in conformation near neutral pH (1). To ensure that we are studying the behaviour of a homogeneous population, the pH dependence of the CD spectrum of DAG-pepsin was investigated (Fig 1). Changes in secondary structure were seen to occur in a narrow range, between pH 6 and 7. The changes were complete within the mixing times of these experiments (5 seconds) and no further changes occurred after overnight incubation (15 hours). Analysis of spectra obtained at extremes of pH in terms of secondary structure (4), indicated that at pH 4.5, DAG-pepsin contained 17±1% alpha helix and 39±2% beta pleated sheet. At pH 7.5, the estimated amount of helix was unchanged, while the extent of beta sheet was reduced to 28±1%.

At both pH values, the secondary structure of the protein was completely destroyed by the addition of 10M urea (Fig 1). However, marked differences were seen at the two pHs when the denaturant was removed. At pH 4.5, removal of urea always resulted in aggregation of DAG-pepsin as evidenced by high levels of light scattering in the solution. The same result was obtained whether the reduction in urea concentration was produced by rapid dilution of a

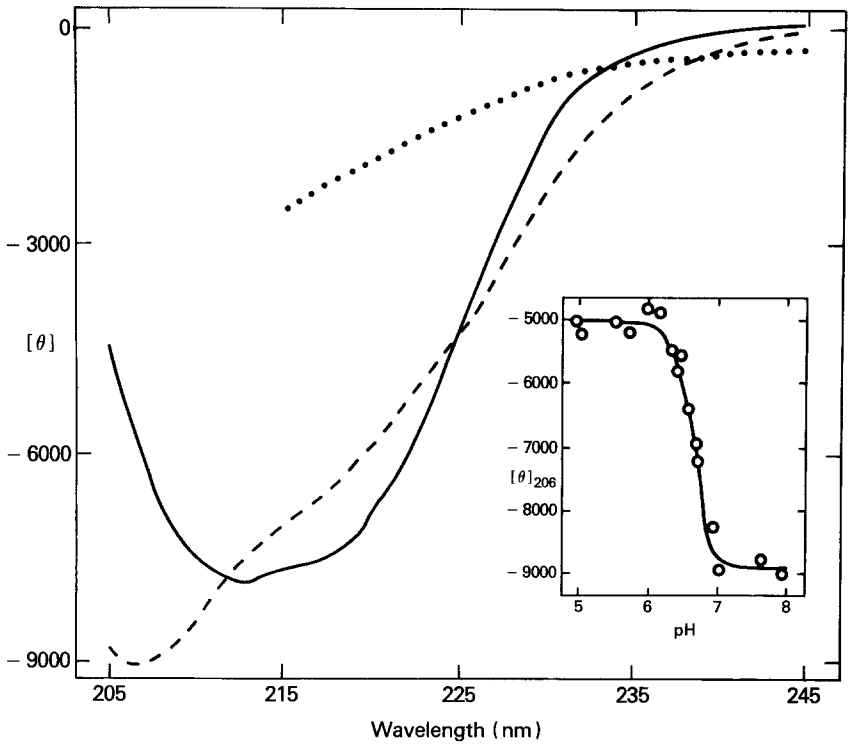

Figure 1. Circular Dichroism Spectra of DAG-pepsin: (——) pH 4.5; (----) pH 8.0; (.....) 10M urea; protein concentration 0.5uM, buffer 0.1M phosphate, 25° C. Inset; pH dependence of the mean residue ellipticity at 206nm.

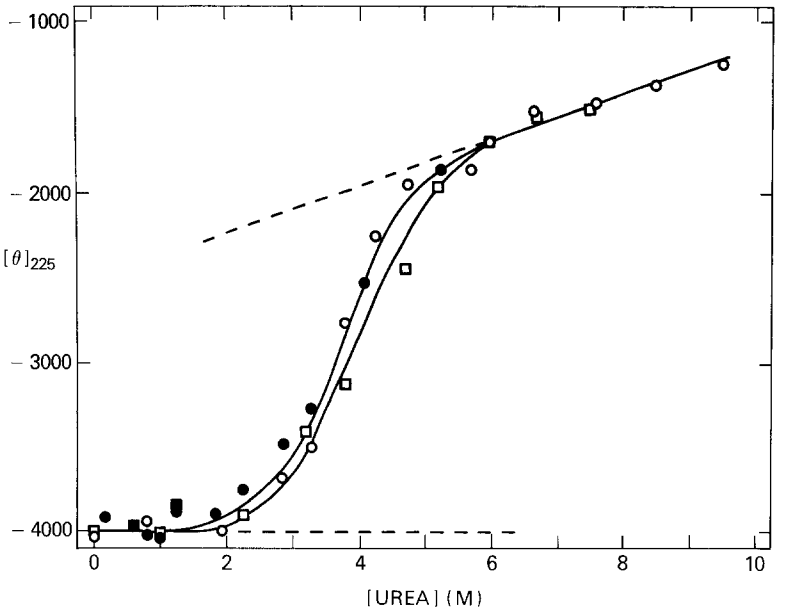

Figure 2. Variation of the mean residue ellipticity at 225nm, of DAG-pepsin (○,●) and pepsinogen (□,■) with urea concentration, 0.1M phosphate buffer, pH 7.5, 25° C. Open symbols, increasing urea concentration; closed symbols, decreasing concentration. The solid lines were fitted to the data as described in the text. The dashed lines represent solvent effects on the ellipticities of the native and unfolded proteins, extrapolated into the transition zone.

concentrated protein solution containing 10M urea into a large volume of pH 4.5 buffer, or by serial dialysis of a dilute solution of unfolded DAG-pepsin, against a series of buffers of decreasing urea concentration. In contrast, removal of urea by rapid dilution, into pH 7.5 buffer, resulted in immediate regain of the original conformation of the protein at that pH, at least as judged by CD. This result was independent of the pH of the unfolded protein solution.

To investigate this phenomenon more closely, equilibrium unfolding and refolding curves were determined for both DAG-pepsin and pepsinogen, at pH 7.5, as described previously (1), except that the measured variable was ellipticity (Fig. 2). In both cases, the experimental results were found to lie on smooth sigmoidal curves, with midpoints close to 4M urea. These curves were analyzed to determine apparent free energies of unfolding, ΔG, and these were expressed as functions of urea concentration as suggested by Pace (5);

$$\Delta G = \Delta G_o - M^*[\text{Urea}].$$

In both cases, good fits to the data were obtained. For pepsinogen, ΔG_o = -3.82 ± 0.35 kcals/mole and M = 1.00 ± 0.09 kcals/ mole^2; DAG-pepsin gave ΔG_o = -4.16 ± 0.42 kcals/mole and M = 1.17 ± 0.12 kcals/mole^2. The stabilities of the proteins were not detectably changed by the addition of twofold molar excesses of pepstatin or of pepsinogen activation peptide (1-16).

Attempts were made to study the kinetics of these reactions by following the changes in CD signal at 225nm as a function of time after jumps in urea concentration. For pepsinogen the results were in good agreement with previous data (2). Changes on unfolding followed a single exponential, $k= 0.03s^{-1}$, in 8M urea , while changes on refolding were biphasic, with 80% of the change occurring within the mixing time (5s), the remainder following a single exponential, $k = 0.01\ s^{-1}$, in 0.16M urea. Under all conditions, the entire signal changes accompanying unfolding and refolding of DAG-pepsin were complete within the mixing time of the experiments.

Discussion The crystal structures of all acid proteases show that their polypeptide chains form four topologically equivalent structural units, which are paired into two domains. These are also present in the precursor, pepsinogen, together with a third amino-terminal activation domain (6). Wetlaufer (7) was the first to suggest that domains could form independent folding units in the formation of protein structure. Much evidence has accumulated in favour of this proposal. For example, Rowe and Tanford (8) interpreted their studies on the folding mechanism of the human immunoglobulin kappa light chain in terms of intermediates on the folding pathway, each having one of its two domains folded. They found no evidence of interaction between the domains. More recently, Adams et al. (9) showed that the two domains of yeast phosphoglycerate kinase could unfold and refold independently, but that the C-terminal domain was stabilised by mutual interactions in the native protein. Privalov and coworkers (10) studied the thermal unfolding of pepsin at a number of pH values by microcalorimetry, finding two distinct transitions. Only the one occurring at higher temperatures, which they ascribed to melting of the C-terminal domain, was reversible. McLachlan (11) has proposed a theoretical folding pathway for pepsin domains, based on their crystal structures.

The present study shows that at pH values above 6, DAG-pepsin undergoes a conformational change to a form which has about 80% of the secondary structure of the native enzyme. At 25° C, this form of DAG-pepsin can be reversibly unfolded at high concentrations of urea (Fig. 2), which overlap the unfolding zone of the intact precursor, pepsinogen. Kinetic studies showed that folding of pepsinogen occurred in two distinct stages; a rapid reaction to form one or more intermediates followed by a slow reaction to produce the native, activatable form (2) . All of the sequence of DAG-pepsin is present in two of the three domains of the zymogen (6). Since its refolding reaction occurs in the same time frame as formation of pepsinogen's folding intermediates, it is highly likely that this neutral structure of DAG-pepsin is present in one or more of them. CD spectra show that this structure differs from that found in native pepsin, (Fig. 1). Since its stability is not affected by the presence of

two tightly binding pepsin inhibitors, it must lack specific binding sites for these two molecules.

Current theories of protein structure place the rate limiting transition state for both folding and unfolding close to the native structure (12). Since the rates of these reactions are so much faster for DAG-pepsin than pepsinogen, this neutral conformation of DAG-pepsin may represent part of an intermediate present before this rate limiting step, on pepsinogen's folding pathway. Thus the two domains of the pepsin portion of pepsinogen are not completely independent folding units. To cross the highest energy barrier to reach their native states, as found in active pepsin, participation of the amino terminal domain of pepsinogen is required. The crystal structure of the zymogen suggests ways in which this may occur (6). For example, the amino terminal residues of pepsinogen fold into the first strand of a large central beta sheet, but during activation they are displaced by the residues which form the amino terminus of pepsin. Perhaps equally important are the large number of specific electrostatic interactions between the amino terminal domain and both domains of the pepsin portion of the molecule. These interactions would not be stable under acid conditions, explaining the inability of the zymogen to fold correctly at pH values below 6. In general, equilibrium protein folding intermediates are present only at low concentrations and kinetic intermediates are present only transiently (12), making investigation of their properties difficult. Characterisation of partially folded forms of protein fragments provides one way to determine structures on the folding pathway.

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