A protein-folding reaction under kinetic control

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SYNTHESIS of α-lytic protease is as a precursor containing a 166 amino-acid pro region[1] transiently required for the correct folding of the protease domain[2–4]. By omitting the pro region in an *in vitro* refolding reaction we trapped an inactive, but folding competent state (I) having an expanded radius yet native-like secondary structure. The I state is stable for weeks at physiological pH in the absence of denaturant, but rapidly folds to the active, native state on addition of the pro region as a separate polypeptide chain. The mechanism of action of the pro region is distinct from that of the chaperonins[5,6]: rather than reducing the rate of off-pathway reactions, the pro region accelerates the rate-limiting step on the folding pathway by more than 10^7. Because both the I and native states are stable under identical conditions with no detectable interconversion, the folding of α-lytic protease must be under kinetic and not thermodynamic control.

Previously, we found that α-lytic protease denatured in 6 M guanidine under nonreducing conditions regained enzymatic activity when the guanidine was removed by dialysis in the presence but not in the absence of the pro region[4]. That the pro region strongly inhibits the native enzyme (inhibition constant, $K_i \sim 10^{-10}$ M (ref. 4)) suggested that it functioned in a late step in the folding pathway. Thus, we reasoned that it might be possible to trap a folding intermediate by removing denaturant in the absence of the pro region. Removal of the guanidine by dialysis resulted in considerable sample aggregation and recovery of less than 4% of the starting activity after addition of the pro region. By contrast, little aggregation occurred when the guanidine concentration was rapidly lowered by dilution. The addition of stoichiometric amounts of a glutathione transferase-pro region fusion protein (GEX–PRO, see ref. 4) to the diluted material resulted in recovery of 74% of the input activity whereas GEX alone had no effect (Fig. 1). This inactive, but folding competent, state of the protease (I) is either directly on the folding pathway or in rapid equilibrium with a conformation on the folding pathway.

A large energy barrier must block the conversion of the I state to the native state in the absence of the pro region. To estimate

TABLE 1 Dependence of folding competence on ionic strength and temperature

	Activity (% $t=0$)	Absorbance at 340 nm
(*a*) Tris concentration (mM)		
5	75	0.008
50	40	0.020
100	26	0.053
200	15	0.094
500	3	0.142
(*b*) Temperature		
4	85	<0.001
24	3.5	0.21
37	0.25	0.23

To investigate the stability of the I state, denatured α-lytic protease was diluted into buffers of varying ionic strength, incubated for 16 h, and then supplemented with GEX–PRO and assayed for refolding. Recovery of activity decreased with increasing ionic strength and increasing temperature. This decrease correlated with an increase in turbidity (A_{340}) suggesting that aggregation accounted for the loss in refolding competence in both cases. The decrease in aggregation at low ionic strength is likely to result from the increased electrostatic repulsion of the highly charged monomers ($pI = 10.2$) due to the loss of counter-ion shielding. The increased stability at low temperature may result from the reduction in the strength of hydrophobic interactions that are likely to mediate aggregation. Methods. *a*, Denatured α-lytic protease in 6 M guanidine HCl was diluted (final concentration of protease 2.5 μM) into the indicated concentration of Tris-Cl plus 100 μM AcProBoro Val and incubated for 16 h at 24 °C. Samples were then supplemented with a twofold molar excess of GEX–PRO and assayed for recovery of enzymatic activity as described in Fig. 1 legend. The absorbance of the solution at 340 nm was also monitored to assess the degree of aggregation of the protease. *b*, Denatured α-lytic protease was diluted into 5 mM KPO_4 pH 8.0, 100 μM AcProBoroVal (final concentration of protease 6 μM) and incubated for 16 h at the indicated temperature. The diluted protease solutions were then treated as in *a*.

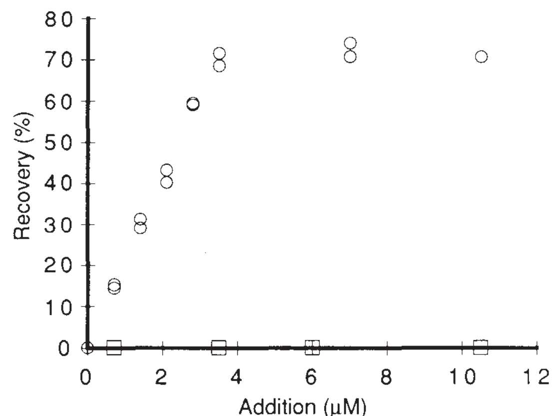
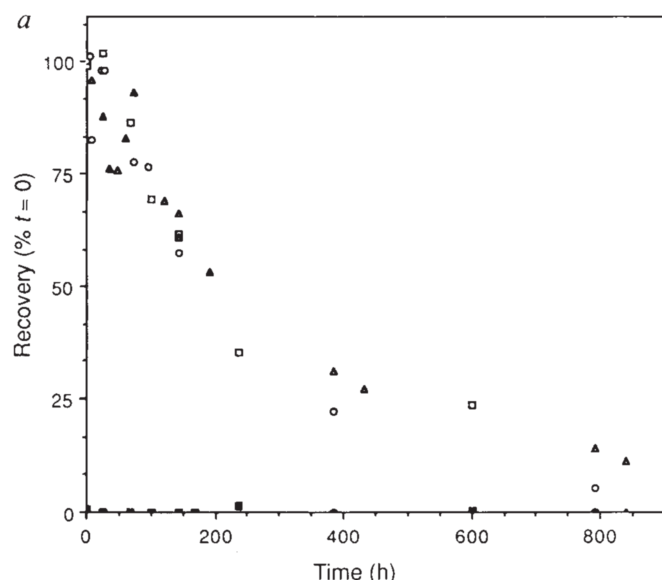


FIG. 1 Specificity and stoichiometry of pro region-dependent conversion of the I state to the native state. The inactive but folding competent state I was prepared by rapid dilution as described below and incubated with either pro region supplied as a glutathione transferase-pro region fusion (GEX-PRO[4], ○) or glutathione transferase alone (GEX, □) as a control. Samples were treated with trypsin to relieve pro-region inhibition and assayed for recovery of protease activity. Trypsin rapidly destroys the pro region and unfolded α-lytic protease species, while leaving the folded enzyme intact[4]. The concentration of the I state determined from the absorbance at 280 nm was 4.4 μM. The maximum amount of native protease recovered was 3.25 μM corresponding to a 74% recovery. The refolding incompetent material may correspond to proteolytic fragments not removed by dialysis or to small aggregates. The observed requirement for stoichiometric amounts of GEX–PRO is probably due to strong product inhibition; the pro region binds the folded enzyme with extremely high affinity ($K_i \simeq 10^{-10}$ M (ref. 4)).

METHODS. α-Lytic protease was purified essentially as described in ref. 9, except that the cation exchange chromatography step was done on S-Sepharose at pH 9.6 with a 50–200 mM salt gradient for elution. Enzyme was dissolved at 200 mg ml^{-1} in 100 mM Tris-Cl pH 8.0 and then denatured by addition of 3 volume equivalents of 8 M guanidine-Cl, 0.67 M glycine pH 3.0. This procedure minimized autolysis at high protease concentrations. The protease was incubated in the 6 M guanidine solution for at least 4 h at 24 °C to ensure complete denaturation. The denatured protein was extensively dialysed (using a 6–8 K cutoff membrane) against 6 M guanidine to remove any fragments from residual autoproteolysis during denaturation. Dilution was carried out by placing a small (5–10 μl) portion of denatured protease solution at the bottom of a test tube and rapidly dispersing the drop by addition of a 200–500-fold excess of buffer lacking guanidine. The dilution buffer contained 100 μM AcProBoroVal, a low-affinity ($K_i = 3$ μM (ref. 13)) α-lytic protease inhibitor, to prevent possible autolysis by residual folded protease. Aliquots of the diluted protease solution were supplemented with GEX or GEX–PRO proteins as indicated and incubated for at least 15 min at 4 °C. To relieve inhibition by the pro region, samples were then treated with trypsin (5 μg) for 10 min at 24 °C. α-Lytic protease activity was then assayed as described in ref. 2. Trypsin does not interfere with the α-lytic protease assay[4].



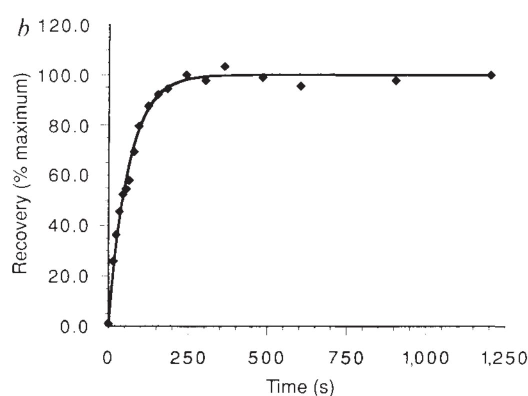
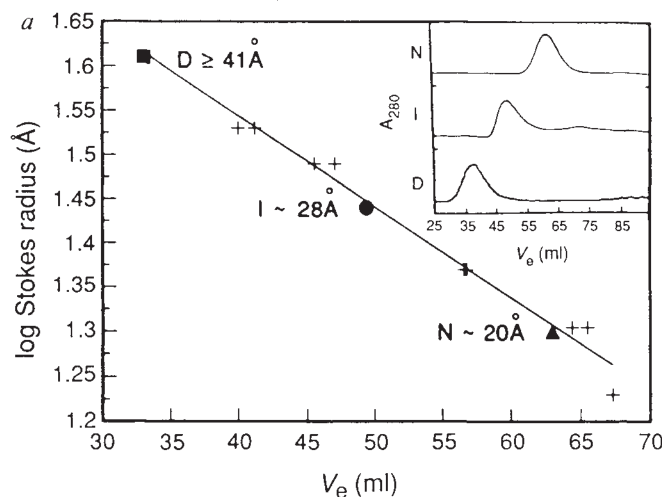


FIG. 2 Kinetics of folding in the absence and presence of the pro region. *a*, To estimate the height of the barrier blocking the folding of the I state to the active enzyme, the I state was prepared as described in Fig. 1, adjusted to a final concentration of 0.3 μM (□), 1 μM (○) or 5 μM (△) and incubated at 4 °C for the indicated time. Samples were then supplemented with a twofold molar excess of GEX (filled symbols) or GEX-PRO (open symbols) and processed as described in Fig. 1. The slow loss of refolding competence with time may result from aggregation, proteolysis, disulphide exchange or other chemical modification. The detection limit in the protease assay is roughly 0.3 nM, corresponding to a recovery of 0.3% of the starting activity in the most concentrated sample (5 μM). *b*, The kinetics of folding in the presence of the pro region were measured by mixing the I state (0.4 μM) with GEX-PRO (0.04 μM) at 4 °C at $t = 0$ and adding trypsin (final concentration 0.7 mg ml^{-1}) at the indicated times to destroy the pro region and any unfolded α-lytic protease. After 10 min at 24 °C, α-lytic protease activity was assayed. Extrapolation of the data to zero time suggests that the lag time for proteolysis by trypsin was <3 s. A plot of a simple exponential fit to the kinetic data is shown (solid line, $k = 0.016\ s^{-1}$). The half time of folding was independent of the intermediate concentration over a 20-fold range (0.4–8 μM) indicating that the apparent dissociation constant of the I state for the pro region is below 0.4 μM.



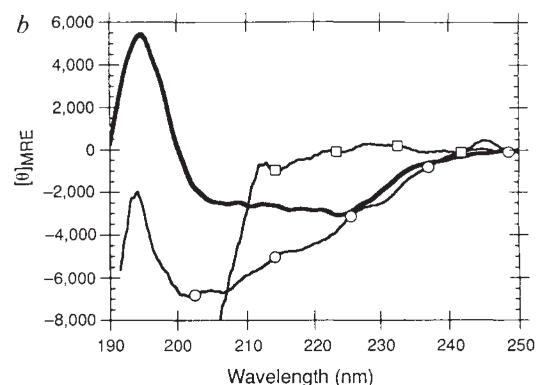


FIG. 3 Structural characterization of the I state. *a*, Gel filtration traces (shown in insert) for the I state, native (N) and denatured (D) forms of α-lytic protease. The hydrodynamic radius of the I state is intermediate between the native and denatured species. The crosses (main figure) indicate positions of molecular mass calibration standards. *b*, Peptide-region CD spectra of N, I and D. The secondary structure content of the I state was estimated by spectral deconvolution:

	% α helix	% β sheet	% β turn	% unordered
Native (bold)	4	75	2	19
I-state (circles)	0	70	3	28
Denatured (squares)	0	6	41	53

Although CD provides a poor measure of β-sheet content, the estimates obtained by spectral deconvolution of the peptide region agree well with values calculated from the X-ray crystal structure[14] using the Kundrot and Richards algorithm[15]: 74% β sheet and 2.5% α helix.

METHODS. *a*, A G-75 Sephadex column (3–80K fractionation range) at 4 °C was used for all sizing data. This resin was selected because it showed minimal interaction with native protease under the low salt conditions required for stability of the intermediate. The degree of interaction of the expanded intermediate with the gel matrix is unknown. Therefore, the apparent molecular mass for the I state must be considered a minimum estimate. The running buffer was 10 mM Tris, 40 mM NaCl pH=8.0 for both N and I. Denatured dialysed protease was prepared as described in Fig. 1 and diluted to 30 mM (I) or 1 M guanidine (D). The 1 M guanidine sample (D) was run in 10 mM Tris, 1 M guanidine-HCl pH=8.0. Higher concentrations of guanidine were not used owing to low flow rates at 4 °C, however CD indicates that the I state is denatured in 1 M guanidine. Stokes radii of the calibration standards were obtained from ref. 16. As Sephadex obeys the principle of universal calibration[17], the calibration in part B should hold for the 1 M guanidine trace. Occasionally fragments were still seen in both I and D traces; presumably deriving from autolysis in the original 6 M guanidine denaturation. Sometimes the intermediate trace also showed a peak size corresponding to protein aggregates. But refolding activity in both I and D always correlated with the 40K and ≥80K peaks. The concentrations of the I state used in gel filtration fall within the range observed to have a linear CD signal at 220 nm indicating the absence of a dimer ⇔ monomer equilibrium at these concentrations. *b*, Native, I and denatured samples were prepared as described above except that they were buffered in 5 mM KPO_4 at pH 7.0 for increased far-ultraviolet transparency. Spectra were taken on a Jasco J500A spectrophotometer at 15 °C using 0.1 mm pathlength. Concentrations used for the peptide spectra were 24.1 μM, 8.9 μM and 7.9 μM for N, I and D. The concentration dependence of the CD signal at 220 nm is linear up to and including the concentrations of I and N used in this study. Deconvolutions of peptide region spectra were done using the Yang algorithm[18]. Native and intermediate spectra were analysed from 240–190 nm. The denatured spectrum was deconvoluted from 240 to 205 nm owing to lack of low wavelength data.

the height of this barrier, the I state was incubated for an extended period of time under conditions which minimize aggregation (Table 1). Although the ability to recover activity in the presence of the pro region slowly decayed with time, large amounts of activity were recovered even after a month of incubation (Fig. 2*a*). By contrast, no activity was recovered in the absence of the pro region in 800 h of incubation. From the detection limit in the protease assay, the first order rate constant for conversion of the I state to the native state is estimated to be $<10^{-9}\,s^{-1}$. Thus, even with the three native disulphide bonds in place, the native conformation does not seem to be kinetically accessible from the I state. On the basis of this data, the free energy barrier for conversion of I to the native state in the absence of the pro region is in excess of 27 kcal mol^{-1}.

The kinetics of folding in the presence of the pro region (Fig. 2*b*) are consistent with a simple single turnover mechanism: $I + Pro \Leftrightarrow I\text{-}Pro \Rightarrow Nat\text{-}Pro$. Strong product inhibition[4] presumably accounts for both the single-turnover kinetics and the observed stoichiometry (Fig. 1) of the refolding reaction. The apparent rate constant for conversion of the I-Pro complex is roughly $0.016\,s^{-1}$. These results indicate that the pro region increases the rate of folding (k) by over seven orders of magnitude ($k(+pro)/k(-pro) \geq 1.7 \times 10^7$).

Structural characterization shows the I state to have properties intermediate between the native and denatured states. By gel filtration (Fig. 3*a*), the I state chromatographs as a single species with an apparent Stokes radius (28.4 Å) between that of native (20.4 Å) and denatured enzyme (37.5 Å). Circular dichroism (CD) spectroscopy (Fig. 3*b*) shows that the I state contains nearly as much secondary structure (70% β sheet) as the native state (N, 75% β sheet) and much more than the fully denatured state (D, 0% β sheet). Aromatic region CD and fluorescence spectroscopy both indicate that the I state has little or no organized tertiary structure (data not shown).

The presence of well defined secondary structure and absence of tertiary interactions indicated by the CD and fluorescence spectra, together with the expanded hydrodynamic radius, are hallmarks of the 'molten globule' or A states of various proteins under non-native conditions[7-10]. But the I state may be the first example of a long-lived 'molten globule'-like conformation of a polypeptide chain under conditions in which the native state is also stable.

In principle, a *trans*-acting factor could promote folding either by decreasing the rate of off-pathway folding reactions or by increasing the rate of a limiting on-pathway reaction. The molecular chaperonins, a ubiquitous class of proteins which are thought to bind to partially folded proteins and prevent irreversible aggregation and misfolding, seem to aid folding through the former mechanism[5,6]. By contrast, the pro region functions by directly stabilizing the rate-limiting folding transition state, thereby increasing the rate of folding by over seven orders of magnitude. The inability of denatured α-lytic protease to fold to the native state in the absence of the pro region is not due to competing off-pathway reactions under the conditions used in the experiments described above: folding competence is retained in the absence of the pro region (Fig. 2*a*).

It is generally assumed that protein folding is under thermodynamic control[11,12]. By lowering the height of a limiting energy barrier, the pro region provides a means to access new regions of conformational space. A 'new' state (the enzymatically active state) is found which has considerably different properties from the low energy state (I) reached in the absence of the pro region. Without the pro region, both states are stable for weeks under identical conditions with no detectable interconversion; hence one of these states must be kinetically trapped.

Perhaps our most important finding is that the energy barriers separating minima in polypeptide chain conformational space can exceed 27 kcal mol^{-1}. If such barriers were present on the folding free energy surfaces of other proteins, large regions of conformational space would be kinetically inaccessible. It would then be conceivable that in such cases the native conformation might be at a local and not a global free energy minimum. □

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RETRACTION

Amyloid plaques, neurofibrillary tangles and neuronal loss in brains of transgenic mice overexpressing a C-terminal fragment of human amyloid precursor protein

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THE paper published under this title[1] has been retracted: see Scientific Correspondence[2].

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