The Mechanism of Folding of Globular Proteins

EQUILIBRIA AND KINETICS OF CONFORMATIONAL TRANSITIONS OF PENICILLINASE FROM *STAPHYLOCOCCUS AUREUS* INVOLVING A STATE OF INTERMEDIATE CONFORMATION

By BARRY ROBSON* and ROGER H. PAIN

Department of Biochemistry, University of Newcastle upon Tyne,
Newcastle upon Tyne NE1 7RU, U.K.

(Received 21 October 1975)

1. The thermodynamically reversible unfolding and refolding of penicillinase between the native and fully unfolded states were followed by using guanidinium chloride as denaturant. 2. The equilibria, studied by optical rotation, u.v. absorption, viscosity and enzyme activity, show the presence of a state of intermediate conformation, termed state H, which is stable at 20°C in 0.8M-guanidinium chloride. 3. The physical properties of this state show that it is slightly expanded with an intrinsic viscosity of $8\text{ ml} \cdot \text{g}^{-1}$, that the 13 tyrosine residues, which are distributed through the primary sequence, are maximally exposed to the solvent and that the helix content is the same as that of the native state. 4. The kinetics of the transition between the native state, state H and the fully unfolded state were followed by u.v. absorption and by optical rotation. They are interpreted as showing that state H lies on the folding pathway between the native and fully unfolded states. 5. The transition between the native state and state H exhibits monophasic unfolding kinetics and biphasic refolding kinetics. This indicates that there must be at least two intermediate states in this process, at least one of which lies on the folding pathway which may also involve cul-de-sac paths. 6. The results are discussed in terms of a mechanism involving rapid stabilization of nucleation regions in a moderately compact but internally solvated structure, with 'native format' [Anfinsen (1973) *Science* **181**, 223–230] secondary structure stabilized by tertiary interaction. The final and rate-limiting step in refolding involves shuffling of these structural elements into the native state. 7. This model is discussed in relation to folding *in vivo*.

It is becoming increasingly clear that the folding pathways of globular proteins can rarely be accounted for in terms of a simple two-state model. Even when such a system is implied by the kinetics, for example lysozyme (Tanford *et al.*, 1966), it is possible to reconcile these kinetics with a much more complex series of consecutive transitions (Tsong *et al.*, 1972). Experimental (Anfinsen, 1973) and theoretical (Scheraga, 1971; Nagano, 1974) studies have emphasized the importance of nucleation steps in which incipient secondary structures, dictated by local interactions, can exist long enough to act as a nucleus on which a very limited number of different conformations can be built up. The discussion of nucleation can be extended to a concept of folding whereby a definite kinetic pathway is followed *in vitro* from the fully unfolded polypeptide chain to the native state, and some experimental evidence sup-

ports this alternative to an unguided search for a free energy minimum (e.g. Wetlaufer *et al.*, 1974; Creighton, 1975). Further support for this approach and delineation of the pathway requires the investigation of conformations that are intermediate in structure between the native and unfolded states. As part of a search for conformational intermediates we have studied the equilibria and kinetics of folding and unfolding of penicillinase from *Staphylococcus aureus* by a variety of techniques. It has been demonstrated that conditions can be defined such that the unfolding of this enzyme is thermodynamically reversible (Robson & Pain, 1976). The presence of a stable state of penicillinase corresponding to neither native nor fully unfolded state has also been reported (Robson & Pain, 1973). In the present paper we present evidence concerning the structure of this state, examine the kinetics of the transition to and from the native state and draw conclusions as to the involvement of this structure in the refolding of penicillinase from the fully unfolded state.

* Present address: Department of Biochemistry, University of Manchester, Manchester M13 9PL, U.K.

Materials and Methods

Staph. aureus exopenicillinase was prepared from the PC1 strain of *Staph. aureus* and purified as described in the preceding paper (Robson & Pain, 1976).

Guanidinium chloride was prepared from ethanol-precipitated guanidinium carbonate (AnalaR grade), recrystallized from benzene/methanol and again from methanol. A 6M solution had $E_{235} \approx 0.2$. Concentrations of guanidinium chloride were checked by refractive-index measurements (Kielley & Harrington, 1960). All guanidinium chloride and penicillinase solutions were made up in 0.1M-KCl/0.01M-sodium phosphate/0.001M-EDTA, pH7.0, and filtered immediately before use.

N-Acetyl-L-tyrosine ethyl ester was obtained as the monohydrate from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.

Difference spectroscopy

Difference spectra were recorded as described in the preceding paper (Robson & Pain, 1976). Kinetic rate constants were obtained by following changes of extinction with time at 286.5nm and plotting the results according to the first-order rate equation.

Solutions left in guanidinium chloride solvent were found to aggregate slowly, as shown by increase in turbidity according to second-order and higher kinetics and by sedimentation velocity. At the lower protein concentrations used for difference spectroscopy (≤ 2 mg/ml) this does not pose a serious problem within the time-scale of the experiment, except occasionally at higher (~ 3 M) concentrations of guanidinium chloride and higher temperatures ($\geq 30^\circ\text{C}$).

Viscosity

Measurements of viscosity were made in a viscometer of the type described by Schachman (1957) with a flow time for water of 360s. Temperature was controlled to within $\pm 0.01^\circ\text{C}$.

Results

Equilibria of unfolding by guanidinium chloride

When the extinction difference $\Delta E_{286.5}/E_{276.5}$ is plotted as a function of guanidinium chloride concentration, a blue shift is observed which is monotonic within experimental error from close to zero to approx. 1M-guanidinium chloride (Figs. 1 and 2). The sign of the dependence of ΔE on guanidinium chloride concentration is then reversed and a linear red shift follows as the concentration of guanidinium chloride is raised further. The only effect of tem-

perature is to increase the slope of the red-shift region of the plot, whereas the initial parts of the curves corresponding to the blue shift are, within experimental error, superimposable.

The occurrence of a blue shift at these wavelengths when a protein is perturbed by a denaturing agent such as urea or guanidinium chloride is usually interpreted in terms of a conformational transition in which aromatic chromophores (in this case tyrosine residues) are transferred from a relatively non-polar protein environment to the solvent environment (Yanari & Bovey, 1960).

The slope of the red shift can be predicted from the perturbation of model compounds by solvents. The red shift of the absorption spectrum for *N*-acetyl-L-tyrosine ethyl ester was measured as a function of guanidinium chloride concentration and the change of $\Delta E_{\text{max}}/E_{\text{max}}$ found to be 0.0180/mol of guanidinium chloride. Combining this result with the 54% exposure of the 13 tyrosine residues in penicillinase (Robson & Pain, 1976), the enzyme in guanidinium chloride should show a red shift of $\Delta E_{\text{max}}/E_{\text{max}} = 0.0097$ /mol of guanidinium chloride when folded and 0.0145/mol of guanidinium chloride when unfolded, assuming 80% exposure in the unfolded state (Tanford, 1970, p. 47) and ignoring slight changes in E_{max} values on change of solvent (Bailey *et al.*, 1968).

The experimental value for penicillinase is 0.02/mol of guanidinium chloride at 20°C (Fig. 2a).

Fig. 1. U.v. absorption spectrum and difference spectrum in 1.0 M-guanidinium chloride of *Staph. aureus* PC1 penicillinase

Buffer for absorption spectrum and for reference solution in the difference spectrum was 0.1M-KCl/0.01M-sodium phosphate, pH7.0. Penicillinase concentration was 2.9mg/ml, and temperature 20°C . For further details see the Results section under 'Equilibria of unfolding by guanidinium chloride'. Arrows indicate the relevant ordinates for the absorption (E) and difference (ΔE) spectra respectively.

Fig. 2. Equilibrium curves for the unfolding and refolding of penicillinase in guanidinium chloride as measured by difference spectroscopy at 286.5nm

Temperature: (a) 20°C; (b) 30°C. Starting solvents for each experiment were: ○, 0.1M-KCl/0.01M-sodium phosphate, pH7.0; Δ, 2M-guanidinium chloride/0.01M-sodium-phosphate, pH7.0. For further details see the Results section under 'Equilibria of unfolding by guanidinium chloride'.

The increased slope of the red shift at higher temperatures is contributed to largely by the more rapid onset of aggregation as the temperature is increased (Robson & Pain, 1976) and the somewhat high slope at 20°C may also be due to incipient aggregation (see also the Materials and Methods section).

In Fig. 2 values of $\Delta E_{286.5}/E_{276.5}$ are plotted for solutions diluted from enzyme dissolved in 2M-guanidinium chloride. Together with the full recovery of enzyme activity on dilution from solutions in guanidinium chloride (Robson & Pain, 1976), these results indicate that the observed conformational transition exhibits full thermodynamic reversibility.

The transition was characterized further by measurements of specific viscosity (Fig. 3). These showed a transition in terms of flow properties for penicillinase over the same concentration range of guanidinium chloride which, when plotted together with the difference-spectroscopy results (Fig. 4), indicates that the change in tyrosine environment is associated with a significant conformational expansion of the protein. The intrinsic viscosity of the enzyme in 0.8M-guanidinium chloride has been measured and has a value of $8.0 \pm 1 \text{ ml} \cdot \text{g}^{-1}$. This is rather lower than the value of $28 \text{ ml} \cdot \text{g}^{-1}$ calculated from the semiempirical relationship between intrinsic viscosity and chain length for a fully unfolded protein chain as described in Section IIIA.2 of Tanford (1968), but supports the conclusion that the protein, after the difference-spectroscopy transition, is in an expanded and solvated state.

The results of treating penicillinase with guanidinium chloride, and following the equilibria by

change of optical rotation, $[\alpha]_{234}$ (Robson & Pain, 1976), are combined with the viscosity and difference-spectra transitions in Fig. 4, experimental 'plateau' regions being used to normalize the transition curves. It is apparent that the enzyme is unfolding in two separate stages, that monitored by difference spectroscopy being almost complete before the transition associated with change in $[\alpha]_{234}$ has begun. The absence of further transitions at higher guanidinium chloride concentration indicates that the enzyme is fully unfolded and may be designated by state U. There is therefore a conformational state characterized by complete solvation of the tyrosine residues but having still the native content of helix as judged by $[\alpha]_{234}$. This is termed state H.

Stability of the native and partially denatured states

The stability of a protein exhibiting an unfolding transition in guanidinium chloride may be calculated by extrapolating the logarithm of the equilibrium constant $K_{eq} = [D]/[N]$, where D is the denatured state existing at the post-transition plateau and N the native state, to zero concentration of denaturant. A semi-empirical extrapolation procedure, based on the free energies of transfer of component groups of a protein between environments in which those groups exist in the native and denatured states, is given by Tanford (1970). The assumption is made that the interaction between exposed regions of the protein and the solvent is equal to the sum of the interactions of the component groups with the solvent.

The appropriate information from studies on model compounds is shown in Table 1.

Fig. 3. Equilibrium curve for the unfolding of penicillinase in guanidinium chloride as measured by specific viscosity

Temperature was 25°C, and penicillinase concentration 30mg/ml. For further details see the Results section under 'Equilibria of unfolding by guanidinium chloride'.

Fig. 4. Equilibrium curves for the unfolding and refolding of penicillinase in guanidinium chloride measured by viscosity (1), difference spectroscopy (2) and mean residue rotation $[m']_{234}$ (3)

The curves are plotted from experimental data in Fig. 3 (1), Fig. 2 (2) and Fig. 1 of Robson & Pain (1976) (3). The curves have been normalized by using the extrapolated plateau regions before and after the transitions as appropriate. The fraction of native conformation (F_N) was calculated at each concentration of guanidinium chloride as $F_N = (\gamma - \gamma_D) / (\gamma_N - \gamma_D)$ where γ is the experimental parameter measured and N and D refer to the native and post-transition states respectively. For further details see the Results section under 'Equilibria of unfolding by guanidinium chloride'.

Table 1. Application of the Tanford (1970) theory of denaturation to *Staph. aureus* exopenicillinase

Relation of free energy of transfer in J/mol to guanidinium chloride concentration by using a polynomial having coefficients m_1 , m_2 and m_3 ; see the text eqn. (1). n , Number of residues in penicillinase.

Side chain or group	m_1	$-m_2$	m_3	n
Alanine	54.4	9.6	0.96	18
Leucine	653	109	9.62	22
Isoleucine*	653	109	9.62	19
Valine*	418	84	6.28	16
Threonine	310	67	4.60	13
Asparagine	962	167	13.8	21
Glutamine	644	105	6.69	4
Methionine	694	100	7.95	3
Phenylalanine	824	50	0	7
Tyrosine	686	167	12.1	13
Histidine	895	172	11.7	2
Proline*	192	33.5	2.51	9
Peptide group	397	63	4.18	256

* Reliable data for free energies of transfer are not available for these residues and have been estimated [see Tanford (1970) and the text].

The coefficients m_1 , m_2 , m_3 are those that satisfy the best fit of the polynomial (Ostle, 1954):

$$y = m_1 \cdot x + m_2 \cdot x^2 + m_3 \cdot x^3 \quad (1)$$

to data provided by Nozaki & Tanford (1970). These data are the free energies of transfer of amino acid side chains [Table 3 of Nozaki & Tanford (1970); J/mol of side chain] and peptide group (see below) between water and guanidinium chloride at 25.1°C. In eqn. (1) $y = -(\text{free energy of transfer in J/mol})$ and x is the molar concentration of guanidinium chloride in water.

Reliable data for the transfer of groups marked by an asterisk (*) in Table 1 to guanidinium chloride are not available. From Tanford (1970), isoleucine is taken as being similar to leucine in this context. An estimate of the values of the coefficients for valine was made by applying ratios of the effects of guanidinium chloride to urea at different concentrations (Nozaki & Tanford, 1970, Table 8), to the data for transfer to urea (Tanford, 1963, 1970): it is assumed that the ratios for valine in these two solvents are similar to those for leucine. Data for transfer of proline to urea solutions was obtained from Tanford (1963, 1970), and the free energy of transfer of the proline side chain at any concentration of guanidinium chloride taken to be the same as that in twice that concentration of urea, which is approximately true of all the hydrophobic side chains in the concentration range of interest. The second and third coefficients for proline are estimated from the

observation that the ratio m_1/m_2 lies in the range 5–7 and the ratio $-m_2/m_3$ in the range of 10–15 for all side chains except phenylalanine.

The coefficients for the peptide group are based on the work of Robinson & Jencks (1965) on *N*-acetyltetraglycine ethyl ester, also quoted by Nozaki & Tanford (1970).

Following Tanford (1970, section VB), the free energy of the native state relative to a reference state at a specified concentration of guanidinium chloride, ΔG_N , was calculated as:

$$\Delta G_N = \Delta G_{N,0} + \sum_i (\Delta \alpha_i) \cdot n_i \cdot y_i \quad (2)$$

where n_i is the number of groups of type i , $\Delta \alpha_i$ the average change in their degree of exposure on a scale from 0 to 1 between the two stages under consideration, and y_i the value taken by eqn. (1) for a group of type i at a specified concentration of guanidinium chloride. $\Delta G_{N,0}$ is a constant representing ΔG_N at zero concentration of guanidinium chloride. This is the free energy of the native state relative to a reference state, here taken as the fully unfolded random coil, and is the parameter of interest to be determined. For native-to-random-coil transitions, Tanford (1970) suggested that $\Delta \alpha_i$ for hydrophobic side chains and peptide groups could be treated as a constant in the approximate range 0.25–0.35, the actual value depending on the protein. Since the solvent-perturbation studies show that approximately one-half of the tyrosine residues are exposed in native penicillinase and in the absence of other information, a value of 0.275 was taken (there are no tryptophan and relatively few phenylalanine side chains). Again, following Tanford (1970), $\Delta \alpha_i$ was taken as zero for all hydrophilic side chains.

From the above equilibrium results, it appears that two states, U and H, are in equilibrium with the native state and it should be possible therefore to calculate the free energies of these states relative to that of the native state at zero guanidinium chloride concentration.

In calculating the free energy of state H relative to the fully unfolded state U at specified concentrations of guanidinium chloride, ΔG_H , the assumption is made that the molecule in state H is a mixture of helical and fully solvated regions (see the Discussion section). The calculation is similar to that for ΔG_N , with appropriate changes in the value of $\Delta \alpha_i$. For the change from helix to coil, $\Delta \alpha_i$ was taken as zero for all side chains (Tanford, 1970). The value of $\Delta \alpha_i$ for the peptide group was determined by assuming the change of exposure of the peptide groups to be given approximately by $\Delta \alpha_i = (\text{average exposure to solvent of groups in random coil}) \times (\text{percentage helix content})$. An average exposure of groups in the random coil of 0.75 (Tanford,

1970) and a helix content of 25% lead to a value of 0.185.

$\Delta G_{N,0}$ and $\Delta G_{H,0}$ were determined empirically by first noting the concentrations of guanidinium chloride at which the midpoints of the corresponding transitions occur (Fig. 5 and Table 2). By using the fully unfolded state U as reference (i.e. the baseline at $\Delta G = 0$ in Fig. 6), the point at which the theoretical plot for the dependence of the equilibrium $H \rightleftharpoons U$ intersects this baseline is obtained from the linear plot of ΔG_H versus logarithm guanidinium chloride concentration for the experimental $[m']_{234}$ transition, i.e. where $\Delta G_H = 0$. With this curve now located on the plot of ΔG versus guanidinium chloride concentration, the point at which it intersects the theoretical plot for the dependence of the equilibrium $N \rightleftharpoons H$ is similarly determined from the plot of ΔG_N versus logarithm

Fig. 5. Apparent equilibrium constants for the unfolding of penicillinase in guanidinium chloride as measured by difference spectroscopy, plotted as a function of the logarithm of guanidinium chloride concentration

Equilibrium constants were calculated by using eqn. (5) in the text. The dependence of y_N on guanidinium chloride concentration could be assumed to be zero over the small relevant range of guanidinium chloride concentration or could be calculated approximately from solvent-perturbation data (see the text). The dependence of y_D on guanidinium chloride concentration could be taken from the experimental data or again calculated (see the text). No significant variation in the slope of the straight line was found with the above procedures owing to the small range of guanidinium chloride concentration involved. For further details see the Results section under 'Stability of the native and partially denatured states'.

Table 2. Characterization of the transition curves for the unfolding and refolding of penicillinase obtained by following different physical parameters y as a function of guanidinium chloride concentration

Transitions are characterized by their midpoints and by the dependence of the equilibrium constants for an assumed two-state system on guanidinium chloride concentration (see Fig. 5).

Measured parameter y	[GuCl] at midpoint of transition	$d \log K_{app}/d \log [\text{GuCl}]$
Spectral perturbation at 286.5 nm	0.5 at 20°C	2.0 at 20°C
	0.7 at 30°C	3.3 at 30°C
Relative viscosity	0.5 at 25°C	2.7 at 25°C
Optical rotation	1.2 at 20°C	5.7 at 20°C
	1.05 at 30°C	4.2 at 30°C

Fig. 6. Free energies of conformational states N and H as a function of guanidinium chloride concentration

The curves are derived from difference spectroscopy and optical-rotation data as described in the text. The fully unfolded state U is the reference state. For further details see the Results section under 'Stability of the native and partially denatured states'.

guanidinium chloride concentration for the experimental $\Delta E_{286.5}$ transition. With both ΔG_N and ΔG_H curves thus located, $\Delta G_{N,0}$ and $\Delta G_{H,0}$ are obtained by extrapolation to zero guanidinium chloride concentrations (Fig. 6).

The assumption made is that the apparent equilibrium constants at the transition midpoints approximate the true equilibrium constants for the corresponding transition. This assumption is justified by the calculation from Fig. 6 that around the midpoint of the states N -to- H transition the state H population is 40 times the size of the state U population, whereas around the midpoint of the states H -to- U transition the state U population is 90 times the size of the state N population. It is found that $\Delta G_{N,0} = -25 \text{ kJ/mol}$ and that $\Delta G_{H,0} = -17 \text{ kJ/mol}$.

Fig. 7. Equilibrium constants, K_{OR} , for the unfolding of penicillinase in guanidinium chloride at 30°C measured from optical-rotation data, plotted as $\log K_{OR}$ versus guanidinium chloride concentration

The curve is non-linear and is derived semi-empirically (see the Results section under 'Stability of the native and partially denatured states').

The semi-empirical curves in Fig. 6 can be checked against experimental results obtained from the full transition curves. When the equilibrium constant from optical-rotation measurements, $\log K'_{OR}$, measured at 30°C where more data are available, is plotted against the concentration of guanidinium chloride (Fig. 7) it satisfies a theoretical curve with $\Delta \alpha_t = 0.25$. Still assuming the value of $\alpha = 0.75$ for random-coil residue exposure, this would be in keeping with a helical content of 33% or with lower helical content and a small proportion of side-chain interactions (see below).

A similar comparison can be made for the behaviour of the equilibrium constant for the transition between states N and H . However, since some of the data are found at concentrations of guanidinium chloride where the transition curves appear to overlap slightly, a correction must be made. If states H and U are again considered to have the same difference-spectroscopic properties, then the apparent equilibrium constants obtained from difference-spectroscopy results will be given as:

$$K'_{Ds} = \frac{[H] + [U]}{[N]} \quad (3)$$

A plot of the predicted curve of $\log K'_{Ds}$ versus guanidinium chloride concentration based thus on Fig. 6 is compared with the experimental data in Fig. 8. Considering the approximations the agreement is good. The deviation of the experimental points from the theoretical curve at the right side of the Figure reflects the uncertainty about the post-

Fig. 8. Equilibrium constants, K_{DS} , for the unfolding of penicillinase in guanidinium chloride at 20°C as measured by difference spectroscopy, plotted as $\log K_{DS}$ versus guanidinium chloride concentration

The experimental points were evaluated by using the gradient calculated for the post-transition region of the equilibrium curve and curve A is the best polynomial of degree three fitted to these points by the method of least squares. For comparison, curve B is that which would fit the points evaluated by using the observed gradient of the post-transition region of the equilibrium curve (see legend to Fig. 5). Curve C is derived semi-empirically from free energies of transfer (see the Results section under 'Stability of the native and partially denatured states').

transition curve, and that at low guanidinium chloride concentration is the region of maximum uncertainty in the experimental difference-spectroscopy measurements.

Kinetics of unfolding and refolding

Difference spectroscopy. Rate studies have been carried out both in the forward and the reverse directions over a range of guanidinium chloride concentrations. Except where stated otherwise, refolding reactions were followed from the denatured state in 2M-guanidinium chloride. The magnitude of the difference-spectrum maximum $\Delta E_{286.5}$ was measured as a function of time, and the reactions were followed to 90–95% of the total change to the value at infinite time, $\Delta E_{286.5, \infty}$. When $\log(\Delta E_{286.5} - \Delta E_{286.5, \infty})$ was plotted against the time at which $\Delta E_{286.5}$ was measured, linear plots consistent with first-order kinetics were obtained for the unfolding reaction (Fig. 9a). That these kinetics are strictly first order (at 2.1M-guanidinium chloride) is confirmed by the absence of any signifi-

Fig. 9. First-order, semi-logarithmic plots of the unfolding and refolding reactions of penicillinase in guanidinium chloride

(a) Unfolding in 0.7M-guanidinium chloride at 20°C; (b) refolding and unfolding in 0.34M-guanidinium chloride at 30°C. The points represent samples taken from continuous experimental traces of $\Delta E_{286.5}$ against time. Difference spectrum quantities refer to values measured at times t and ∞ respectively. A constant is added to bring the plots on to the same ordinate scale (see the Results section under 'Kinetics of unfolding and refolding').

cant change in the slope of such plots over a tenfold change in protein concentration (Table 3). For the refolding reaction, however, biphasic plots were obtained similar to those of Simpson & Kauzmann (1953) for ovalbumin, Schechter *et al.* (1970) for

Table 3. Apparent first-order rate constants for the unfolding of penicillinase at 2.1 M-guanidinium chloride, 20°C, pH 7.0, as observed by u.v.-absorption change

Two separate determinations were performed at each protein concentration.

Protein concn. (mg/ml)	$10^3 \times$ Rate constant (S^{-1})
1.26	5.8, 5.0
0.25	4.7, 5.0
0.12	5.5, 4.7

staphylococcal nuclease, and Ikai & Tanford (1971) and Ikai *et al.* (1973) for cytochrome *c*. A representative semi-logarithmic plot is shown in Fig. 9(b).

The slope of the semi-logarithmic plot of the unfolding reaction gives the apparent rate constant for this reaction. When the logarithms of the apparent rate constants are plotted against the logarithm of the concentration of guanidinium chloride (Fig. 10), the data lie on a continuous curve with a marked change of slope at between 0.4 and 0.5 M-guanidinium chloride. The data to the left-hand side of this 'elbow' do not show any significant dependence on the concentration of guanidinium chloride. Above 0.5 M-guanidinium chloride the slope has a gradient of 2.0 at both 20° and 30°C. The curves for 20° and 30°C are displaced vertically with respect to one another, but not horizontally to any significant extent. This is consistent with the approximately zero enthalpy for the major transition between 20° and 30°C deduced from the equilibrium data. The vertical displacement corresponds to an activation energy of 100 kJ/mol for both unfolding and refolding rate constants.

To see whether the comparatively slow kinetics observed by change in optical absorption accounted for the total change measured in the equilibrium difference-spectroscopy experiments, zero-time values of $\Delta E_{286.5}$ were calculated by extrapolation of the first-order kinetic plots. For the unfolding reaction these values were consistent, within $\pm 6\%$ standard deviation, with the values of $\Delta E_{286.5}$ for the native protein in that solvent as obtained by extrapolation from solvent-perturbation studies.

After refolding of protein from 2 M-guanidinium chloride and extrapolation of the first linear phase of the kinetic plots, the mean value of $\Delta E_{286.5}$ at zero time is close to the post-transition slope of $\Delta E_{286.5}$ calculated from solvent-perturbation studies, although the uncertainty is greater than for the unfolding reaction. This uncertainty is connected with the aggregation of the unfolded protein at concentrations of guanidinium chloride above the transition (Robson & Pain, 1976).

Thus the results are consistent with the observed kinetics of unfolding and refolding accounting for

Fig. 10. Apparent rate constants for the unfolding and refolding of penicillinase as a function of guanidinium chloride concentration and temperature

O, k_{app} for the unfolding reaction, and Δ , k_{app} for the refolding reaction (min^{-1}). Kinetics were followed by $\Delta E_{286.5}$. (See the Results section under 'Kinetics of unfolding and refolding'.)

the complete transition described by changes in optical absorption and viscosity. This confirms also that state H has similar difference-spectroscopic properties to the fully unfolded state at 286.5 nm.

Optical rotation. The time-dependence of change of optical rotation at 234 nm was followed at 27°C by using a Cary model 60 instrument. The unfolding of penicillinase from 0.1 M-KCl, pH 7, to 2 M-guanidinium chloride and above was slow and similar in rate to the kinetics measured by difference spectroscopy. The rates of unfolding from 0.8 M- to 2.5 M-guanidinium chloride and refolding from 2 M- to 0.8 M- and to 0.1 M-guanidinium chloride were too fast to be measured, being complete within 30 s.

Treatment of kinetic data from measurements of difference spectroscopy

The kinetics for unfolding and refolding of penicillinase have been treated by the approach of Ikai & Tanford (1971, 1973). The simplest rate

equation for describing a biphasic first-order rate curve is:

$$\frac{y_t - y_\infty}{y_0 - y_\infty} = P \cdot e^{-\lambda_1 t} + (1 - P) \cdot e^{-\lambda_{II} t} \quad (4)$$

where y_t , y_0 and y_∞ are the values of an experimental time-dependent parameter such as optical absorption at times t , zero and infinity respectively. λ_1 and λ_{II} are macroscopic first-order rate constants obtainable (see, for example, Frost & Pearson, 1953) from the experimental progress curves. P is a constant which may normally be taken to indicate the proportion of the total change in parameter y associated with the term $P \cdot e^{-\lambda_1 t}$. In general, values of P for the forward and reverse reactions are not identical. For a two-stage reaction mechanism such as $N \rightleftharpoons X \rightleftharpoons D$, values of λ_1 and λ_{II} will be independent of the direction in which the kinetics are measured, since the observed apparent rate constants are related to the microscopic rate constants by $\lambda = k_f + k_r$.

To obtain information about the mechanism for such reactions, it is essential to measure the kinetics in both forward and reverse directions in order that the number of experimentally measurable variables may equal the number of unknown quantities (see Table 1 of Ikai & Tanford, 1973). A further constraint is that the kinetics must be measured under conditions within the transition, since the equations relating macroscopic to microscopic variables include the apparent equilibrium constant K_{app} for the reaction:

$$K_{app} = \frac{y_{eq} - y_N}{y_D - y_{eq}} \quad (5)$$

where y_N , y_D and y_{eq} are the values of the experimental parameter corresponding respectively to the native and denatured states and to the equilibrium value at the defined concentration of guanidinium chloride. This places greater demands on the precision of the experimental measurements, as $y_{eq} - y_0$ will

be smaller than for reactions followed under conditions outside the transition.

Adopting the convention that $\lambda_1 > \lambda_{II}$, then, for the penicillinase system, λ_1 corresponds to the apparent rate constant of the unfolding reaction. The kinetics of unfolding and refolding for penicillinase, in various concentrations of guanidinium chloride and at 20° and 30°C, have been analysed by fitting the rate equation (eqn. 4) to the experimental reaction profiles, by using least squares as the criterion of goodness of fit. The monophasic unfolding kinetics cannot be described in terms of a single macroscopic rate constant λ , since at least two such constants are required to describe the refolding reaction and λ_1 and λ_{II} must apply to each direction. Thus the constant for the unfolding reaction, P_{unf} , must take the value unity so that λ_{II} makes no contribution to the unfolding kinetics. The parameters for this particular system can also be obtained by measuring the value of λ_{II} directly from the refolding curve when t is sufficiently large and hence calculating λ_1 and the constant for the refolding reaction, P_{ref} , from the same curve (cf. Ikai & Tanford, 1973), or by measuring the value of λ_1 from the unfolding curve and using this to calculate λ_1 and P_{ref} from the refolding curve. Fig. 10 and Table 4 show that the values of λ_1 obtained by these two approaches are in good agreement. At low concentrations of guanidinium chloride, where λ_1 cannot be obtained from the unfolding kinetics owing to the small overall optical change, λ_{II} may be measured directly from the refolding plot as above or, alternatively, be calculated by using assumed values of λ_1 extrapolated from the dependence of λ_1 on guanidinium chloride concentration. The results show that the value of λ_{II} is less reliable when calculated indirectly, although the value of P_{ref} is relatively insensitive to the method of calculation.

P_{unf} or P_{ref} may be regarded as the normalized fractions of the total optical change associated with each exponential term. The values for P_{ref} show that

Table 4. Kinetic parameters at 20° and 30°C for the unfolding and refolding of penicillinase observed by u.v. absorption change

For definitions of λ_1 , λ_{II} and P_{ref} , see the text and eqn. (4).

Temperature	[GuCl] (M)	Directly measured parameter	$10^4 \times \lambda_1$ (s ⁻¹)	$10^5 \times \lambda_{II}$ (s ⁻¹)	P_{ref}
20°C	0.10	λ_{II}	2.8	5.0	0.41
	0.10	λ_1	2.7	2.5	0.42
	0.27	λ_1	2.7	4.8	0.34
	0.36	λ_1	2.8	6.2	0.40
30°C	0.12	λ_{II}	10.0	9.5	0.52
	0.12	λ_1	13.3	1.5	0.51
	0.22	λ_{II}	13.3	9.3	0.50
	0.22	λ_1	13.3	8.3	0.51
	0.34	λ_1	13.3	25	0.41

the terms in eqn. (4) make approximately equal contributions to the total optical change on refolding.

Interpretation of the kinetics

From the equilibrium results, where the change in optical absorption is shown to lead to a partially denatured state H, the above kinetics can be seen to relate to the transition between state N and state H. Following still the treatment of Ikai & Tanford (1973), the simplest models to explain biphasic kinetics are:

and

where X is some conformational state whose spectral properties are intermediate between those of states N and H.

Provided that the value of the apparent equilibrium constant K_{app} (eqn. 5) is positive in sign, and there is no experimental precedent to the contrary, the kinetic criterion for mechanism (I) is that $P_{unf.}/P_{ref.} < 0$. This is not the case for penicillinase and indeed has not been found to be so for any of the proteins whose kinetics have so far been studied in detail (Ikai & Tanford, 1971; Ikai *et al.*, 1973). The criterion for mechanism (II) to hold is:

$$\frac{P_{unf.}}{P_{ref.}} = \frac{\lambda_{11} + P_{unf.}(\lambda_1 - \lambda_{11})}{\lambda_1 + K_{app.}(1 - P_{unf.})(\lambda_1 - \lambda_{11})} \quad (6)$$

The experimental constraint that $P_{unf.}$ must be equal to unity in order to explain the monophasic unfolding kinetics makes eqn. (6) invalid and hence rules out the simple 'off-pathway' intermediate mechanism (II). Similarly, an attempt to explain the kinetics by the model $N \rightleftharpoons U \rightleftharpoons H$ is ruled out by the experimental finding that $P_{ref.} \approx 0.5$, since only the equilibrium between states N and U will be seen by absorption spectroscopy.

The remaining three-component mechanism:

can also be excluded by the need for the ratio $P_{unf.}/P_{ref.}$ to be positive, where:

$$\frac{P_{unf.}}{P_{ref.}} = -\frac{1}{K_{app.}} \cdot \frac{\gamma_2 - k_{12}\lambda_1}{\gamma_2 - k_{32}\lambda_1} \quad (7)$$

and $\gamma_2 = k_{12}k_{31} + k_{12}k_{32} + k_{13}k_{32}$. According to experiment, $(1 - P_{unf.}) \approx 0$ (see above), so that $(\gamma_2 - k_{12}\lambda_{11}) \approx 0$. Since $\lambda_1 > \lambda_{11}$, then $(\gamma_2 - k_{12}\lambda_{11}) < 0$ and

moreover $(\gamma_2 - k_{32}\lambda_1) < 0$, since $k_{32} > k_{12}$ in order to explain monophasic unfolding and biphasic refolding kinetics. This makes $P_{unf.}/P_{ref.} < 0$, which is contrary to experiment.

Thus a minimum of four components is required to account for the experimental kinetics of the transition between states N and H. The mechanism in which all time-intermediate states are on a cul-de-sac, i.e.:

can be excluded on the basis of the equations of Ikai & Tanford (1973). The kinetics of a system involving four components will be represented (cf. eqn. 5) by the relation:

$$\frac{y_t - y_\infty}{y_0 - y_\infty} = P_1 e^{-\lambda_1 t} + P_2 e^{-\lambda_2 t} + P_3 e^{-\lambda_3 t} \quad (8)$$

To account for the monophasic penicillinase-unfolding kinetics it is necessary that $P_{1,unf.} = 1$, and $P_{2,unf.} = P_{3,unf.} = 0$ and, for the biphasic refolding kinetics, $\lambda_2 \approx \lambda_3$.

From eqn. (55) of Ikai & Tanford (1973):

$$\sum_s \lambda_s P_{s,unf.} = \frac{1 + K_{app.}}{K_{app.}} \cdot k_{14} \quad (9)$$

it follows that, if $P_{1,unf.} = 1$, then:

$$\lambda_1 = \frac{1 + K_{app.}}{K_{app.}} \cdot k_{14} \quad (10)$$

Eqn. (57) of Ikai & Tanford (1973) states that the ratio of the forward and reverse P values is given by:

$$\frac{P_{s,unf.}}{P_{s,ref.}} = \frac{k_{14}}{K_{app.}(\lambda_s - k_{14})} \quad (11)$$

For penicillinase (Table 4), $P_{1,unf.}/P_{1,ref.} \approx 2 = k_{14}/K_{app.}(\lambda_1 - k_{14})$, which is incompatible with the expression for λ_1 from eqn. (10) above. Thus the kinetics for the forward and reverse transitions between states N and H do not support a four-state mechanism where both time-intermediate states lie off the folding pathway. Although it is not possible by further analysis to distinguish between models:

and

it can be concluded that the transitions between states N and H must involve at least two kinetically observable time-intermediate states, of which at least one will lie on the folding pathway.

Discussion

The partially denatured state H

Comparison of the equilibrium unfolding curves shown by difference spectroscopy, viscosity and enzyme on the one hand, with that given by optical rotation at the Cotton-effect trough in the other, shows that there are at least two quite separate transitions involved in the equilibria between the native state, N, and the fully unfolded state, U, of penicillinase in the presence of guanidinium chloride. The difference-spectroscopy transition is nearly complete before the optical rotation transition begins (see Fig. 4). Thus a state H possessing an intermediate conformation exists and is expanded and solvated as shown by the increased intrinsic viscosity and by the complete perturbation of the 13 tyrosine residues. The distribution of tyrosine residues throughout the sequence (Ambler & Meadway, 1969; Ambler, 1975) makes it unlikely that the two transitions reflect two domains (Wetlaufer & Ristow, 1973) of the protein unfolding sequentially. The fitting of the experimental points on the unfolding curves to the theoretical curves constructed on the basis of the existence of a single domain (Figs. 7 and 8) (Tanford, 1970; Rowe & Tanford, 1973) again implies that both transitions reflect co-operative transitions each involving the greater part of the whole molecule.

In the absence of denaturant, state H is stable with respect to the fully unfolded state by 17 kJ/mol and unstable with respect to the native state by 8.4 kJ/mol. States N and H contain a similar number (approx. 65) of helical residues (Robson & Pain, 1976), which leads to a value of -260 J/mol of residue for the free energy of helix formation per residue in state H. This is consistent with values obtained from polymers of glutamic acid (-440 J/mol) and lysine (-335 J/mol) obtained by Hermans (1966), but not with the estimate for the leucine residue (-3.5 kJ/mol) by Miller & Nylund (1965). Nevertheless, it is reasonable to expect a smaller free-energy change than is predicted from helix-favouring residues alone, since the overall free energy could be minimized by pulling some coil-favouring residues into the helical conformation.

On the other hand, Scheraga (1971) has concluded, on the basis of experimental parameters for helix-coil transitions in artificial polypeptides, that the time-averaged probability of finding helices of the length that occur in native proteins is very low. This is a consequence of the observed low magnitude of the co-operative parameter (Lifson & Roig, 1961) in such transitions. Singhal & Atassi (1970) have shown that polypeptide sequences that are helical in the folded protein exhibit a much lower helix content when excised and placed in an aqueous environment. One possible conclusion therefore is that the helical

regions of state H are very long. The minimum length of a sequence that spends 90% of its time in the helical conformation depends on the amino acids in it; for example, at 20°C it is between 50 and 100 for polyleucine and about 500 for polyalanine (Scheraga, 1971). It is well known (see, for example, Scheraga, 1971) that uncharged polyglutamic acid, polyaspartic acid and polylysine form stable helices in water, and Yaron *et al.* (1971) have shown that chains of polylysine 50 residues long are approx. 90% helical. State H could therefore be stable if all the helix was in one or, at most, two helical sequences with helix-coil transition properties similar to those of lysine.

An alternative possible implication is that a much larger number than 50-80 residues are spending much less time in the helical conformation, as either a small number of large sequences or a large number of small ones. For example, Yaron *et al.* (1971) found that chains of polylysine 20 residues long spend about half their time in the helical conformation.

Although it cannot be proved at the moment that the helical regions of the polypeptide backbone of state H correspond to those of the native state, the strong correlation between residue type and helical backbone conformation (e.g. Lewis *et al.*, 1970; Robson & Pain, 1971, 1974) makes it unlikely that a substantial number of residues would be helical in one species and non-helical in the other. Lewis *et al.* (1970) have successfully used theoretically predicted probability profiles of denatured proteins to account for much of the secondary structure observed in native proteins by X-ray crystallography. In this prediction the helical regions of the denatured state corresponding to those of the native state spend only 1-10% of their time in the helical conformation because of the low magnitude of the co-operativity parameter used in the theory. Scheraga (1971) has proposed that these helical regions provide nucleation centres for the specific native structure of a protein, after which the helical regions are further stabilized by interactions between residues that are not near-neighbours in the primary sequence. This proposal would be consistent with the findings of the present work and with the interpretation that a limited number of tertiary interactions are stabilizing the helical regions of state H. The fast kinetics found for unfolding and refolding of state H to and from the fully unfolded state are compatible with such minimally stabilized secondary structure in a polypeptide chain (e.g. Burke *et al.*, 1965).

Experimental evidence for the stabilization of helices by restricted tertiary interaction is provided by the increase in helical content in the myoglobin peptide encompassing the two helical regions compared with the helical content of the two separated

regions (Singhal & Atassi, 1970). Poland & Scheraga (1965a,b) have also shown that helices can be stabilized by inter-helical hydrophobic interactions, leading to sharper helix-coil transitions.

The transition between states H and N

The kinetics of folding and unfolding between state H and the native state are more complex than can be explained by a simple two-state system. The results have been interpreted according to the treatment of Ikai & Tanford (1973) as signifying the occurrence of two or more intermediate conformational states of which some may not be on the direct folding pathway. This suggests that the polypeptide chain may not fold directly from states H to N, but explores a limited number of lower-energy conformational cul-de-sacs before finally settling into the native state.

From the dependence of the forward, $k_{\text{unt.}}$, and reverse, $k_{\text{ref.}}$, rate constants on guanidinium chloride concentration (Fig. 10) where $d\log k_{\text{unt.}}/d\log[\text{GuCl}] = 2$ and $d\log k_{\text{ref.}}/d\log[\text{GuCl}] = 0$ the activated intermediate state can be postulated as follows:

where the activated state P^*Gu_2 containing 2 mol of preferentially bound guanidinium chloride (cf. Tanford *et al.*, 1966) must be very similar to state H in terms of exposure to solvent. Further, analysis of the transition between states H and U, monitored by optical rotation at 234 nm, leads to the relation $K_{\text{OR}} \propto [\text{GuCl}]^2$ for the equilibrium constant, confirming that state H is only partially exposed to solvent as compared with the random-coil polypeptide. From the model of state H proposed above, the activation energy of 100 kJ/mol between states H and P^* , which corresponds numerically to the breaking of about five hydrogen bonds, will therefore derive from the rupture of a relatively small number of interactions that are involved in stabilizing regions of secondary structure. The similar activation energy on going from states N to P^* will, on the same grounds, correspond to a larger number of hydrogen bond and van der Waals interactions being broken, but offset by a hydrophobic interaction enthalpy of opposite sign.

This model receives further support from the entropies of activation between state P^* and states N and H respectively, calculated by applying the transition-state theory (see, e.g., Tanford *et al.*, 1966). The entropy of the activated state is higher than that of both N and H states by +111 and +30 J/degree per mol respectively.

The picture emerges of a folding transition from states H to N that involves the breaking and making of stabilizing interactions, which allows a shuffling-round of preformed secondary-structure elements,

into overall lower-free-energy states. After some exploration of lower-free-energy states that lie off the main folding pathway, a final low- or minimum-free-energy state is attained, which includes the helical regions stabilized in state H and in which close, globular packing is attained. The participation of regular structures other than helix awaits analysis of state H by circular dichroism.

Is state H an intermediate?

The remaining question about state H is its role, if any, in the actual folding mechanism of the protein. Its existence shows the possibility of occurrence of stabilized nucleating helices for a ribosome-synthesized polypeptide and thus the reasonableness of proposing a pathway involving similar species. However, the kinetics of the forward and reverse reaction between the native and fully unfolded states suggest that state H may indeed lie on the folding pathway from states U to N. The relevant experimental results are the relative rates of change of the optical-rotation ($[m']_{234}$) and optical-absorption ($\Delta E_{286.5}$) parameters. On unfolding from the native state, both change at similar rates, whereas on refolding $[m']_{234}$ changes more rapidly, by at least three orders of magnitude, than $\Delta E_{286.5}$. Further, the change in $[m']_{234}$ on going from 0.8 to 2.5 M-guanidinium chloride is also very fast. Recalling that there is essentially no change in $[m']_{234}$ between states N and H and no change in $\Delta E_{286.5}$ between states H and U, the simple formulation:

satisfies these experimental findings, with the conformational expansion ($\Delta E_{286.5}$) between states N and H being rate-limiting.

Qualitative examination of an alternative scheme:

shows that it could satisfy the unfolding kinetics between states N and U provided that state H does not become significantly populated. It cannot, however, satisfy the rapid rate of change in $[m']_{234}$ that occurs on going from 0.8 to 2 M-guanidinium chloride. For refolding k_{-2} must be large and k_{-1} large with respect to k_1 in order that the rate constant for refolding as measured by optical rotation change, $k_{\text{OR,ref.}}$, may be much greater than that measured by difference spectroscopy, $k_{\text{DS,ref.}}$. Since the dependence of the rate constants on guanidinium chloride concentration is not large (see Fig. 10) k_{-1} would still be large with respect to k_1 at zero guanidinium chloride concentration and the protein would therefore be left in state H.

A second alternative model:

is also excluded by the demand that $k_{\text{ORD, ref.}} \gg k_{\text{DS, ref.}}$. A triangular scheme:

requires that for unfolding the rates from states N to U and from states N to H be the same. For refolding, the flux from states U to N must be almost, if not entirely, through state H alone. Thus if there were two alternative pathways of refolding, state H would lie as an intermediate on that one chiefly involved in refolding.

The situation is actually more complex than these cases, in that the optical-absorption kinetics indicate the presence of one or more additional states associated with the states $N \rightleftharpoons H$ transition (see above). Since, however, the equilibrium-transition curves are separate, these additional states will not alter the above conclusions. When rate constants can be obtained for the changes in backbone conformation it will be possible to extend this analysis by computer modelling. At the present time it is reasonable to conclude that there is no simple alternative to the hypothesis that the expanded, helical state H lies on the refolding pathway from fully unfolded to native state.

Generality of this model

The question arises as to whether the above model could form the basis of a general pattern for the folding of globular proteins. A similar, though less marked, separation of transitions reflecting different phases of unfolding has been reported for carbonic anhydrase by Wong & Tanford (1973). A similar intermediate state has been postulated for the same protein to explain the kinetic and thermodynamic behaviour on refolding from the acid denatured state (Flanagan & Hesketh, 1974). Lumry & Biltonen (1969), on the basis of a heat capacity of activation, proposed a mechanism for the unfolding of chymotrypsinogen in which the activation step involves a swelling of the protein. Small perturbations of the lysozyme structure are seen by n.m.r. (nuclear magnetic resonance) before complete unfolding of the structure (Bradbury & King, 1969), but techniques that monitor backbone conformation or strongly hydrophobically bonded aromatic groups show nothing but a co-operative two-state transition for lysozyme (Tanford *et al.*, 1966, 1973). Similarly, Benz & Roberts (1975) have shown by n.m.r. that ribonuclease unfolds in a less co-operative fashion than is suggested on the basis of other techniques, and the existence of metastable folding intermediates is supported for staphylococcal nuclease from

n.m.r. studies (Jardetsky *et al.*, 1971) and by the use of site-specific antibodies (Anfinsen, 1973). The present findings on penicillinase, however, constitute the first study of a stable intermediate species.

In general (cf. Tanford, 1970), the observed folding and unfolding equilibria of globular proteins do not exhibit such stable partially denatured states. This need not necessarily mean that such structures are not normally involved in the folding pathways of globular proteins. Other proteins examined so far by the same analytical technique possess a much higher stabilizing energy in the absence of denaturant, e.g. between -42 and -63 kJ/mol for hen's-egg-white lysozyme at pH 6.0–7.0, -44 kJ/mol for ribonuclease at pH 6, and between -42 and -84 kJ/mol for β -lactoglobulin at pH 3 (see Tanford, 1970, Section VIII, for discussion). From the theoretical basis of the plot of ΔG versus guanidinium chloride concentration (Fig. 6), it can be shown that both $\Delta G_{H,0}$ and $\Delta G_{H/\text{guanidinium}}$ chloride concentration increase with the number of helical residues in a molecule. Because of this the midpoint of the transition between a state such as H and the random coil is rather less dependent on the protein concerned, and indeed would occur at approximately the same concentration of guanidinium chloride for proteins with the same average free energy of helix formation per helical residue. If, however, a protein were to undergo a transition involving a major structural expansion well above this concentration of guanidinium chloride, then there would be no demonstrable H intermediate because at no concentration of guanidinium chloride would state H have the lowest free energy (see Fig. 6).

This model offers an approach towards understanding the folding of proteins *in vivo*. As the polypeptide chain comes off the ribosome the incipient formation of helical regions could be stabilized by interactions in a continuously evolving, collapsed structure similar in nature to state H. This would preserve the newly formed polypeptide from the irreversible intermolecular interactions that can occur, as in penicillinase, between unfolded species.

We thank Mr. Allan Robinson, Mrs. Moira Gambie and Mr. Bob Nicholson for skilled technical assistance, and Miss Elizabeth Carrey for the intrinsic viscosity measurement. The work was supported by a Wellcome research training grant to B.R. and by the Science Research Council.

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