TEMPERATURE-DEPENDENCE OF THE KINETICS OF FOLDING OF CHYMOTRYPSINOGEN A

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Received 22 March 1976

1. Introduction

Recently, Brandts et al. suggested that a slow cis–trans isomerization around peptide bonds in unfolded proteins may be important for the kinetics of folding and unfolding of globular proteins [1]. This made a reevaluation of previous kinetic results necessary [2–4]. Using a minimal isomerizamodel with two unfolded states of identical optical properties gives a good quantitative description of the equilibrium and kinetics of the reversible unfolding of chymotrypsinogen A over a wide temperature range, if the change in specific heat is associated with the refolding rate constant.

2. Results

2.1. *Minimal isomerization model*

The simplest model, which incorporates slow isomerization of peptide bonds in the unfolded protein, but otherwise shows all characteristics of a highly cooperative transition between two states, is given by

$$N \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} D \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} D' \qquad (1)$$

where N denotes the native state, D the unfolded state with all peptide bonds in their native conformation, and D′ the unfolded state, where one or more peptide bonds have undergone an isomerization, e.g. from a trans- to a cis-conformation. D and D′ are expected to have very similar or identical optical and energetic properties, but may show a different hydrodynamic or electrophoretic behaviour. Using only one state for D′, with *overall* rate constants for isomerizations k_{23} and k_{32}, is certainly a drastic simplification. But otherwise, extremely complex models have to be considered. For example, with only 10 peptide bonds undergoing an isomerization 100 different states of the unfolded molecule have to be taken into account. Without knowing more about such steps in protein chains the simplification appears to be justified at present. The rate constant for the unfolding is k_{12} and for the refolding k_{21}.

Such a model leads to two relaxation times τ_1 and τ_2 after a perturbation of the equilibrium which are given by [5]:

$$1/\tau_1 = (a + \sqrt{a^2 - 4b})/2 \text{ and} \qquad (2)$$
$$1/\tau_2 = (a - \sqrt{a^2 - 4b})/2$$
$$\text{with } a = k_{12} + k_{21} + k_{23} + k_{32} \text{ and}$$
$$b = k_{12}(k_{23} + k_{32}) + k_{21}k_{32}$$

Depending on the relative magnitude of the different rate constants several simplifications are possible, but usually this general equation will be necessary for describing the kinetics.

For calculating the temperature dependence of the relaxation times the activation energies E_i for the rate constants k_i have to be considered. This gives four equations of the type

$$\ln k_i = -E_i(1 - T/T^0)/R \cdot T + \ln k_i^0 \qquad (3)$$

with T as the absolute temperature, T^0 a reference temperature where $k_i = k_i^0$ and R the gas constant.

From calorimetric measurements it is known that

the unfolding of globular proteins is accompanied by a relatively large change of the specific heat [6,7]. With the assumptions that the change of the specific heat ΔC_p is temperature independent [7] and only associated with the refolding rate constant k_{21}, the activation energies E_{12}, E_{23} and E_{32} will be constant, while the activation energy for the folding shows a temperature dependence according to:

$$E_{21} = \frac{\Delta C_p\,(T \cdot \ln(T/T^0) - T + T^0)}{(1 - T/T^0)} \qquad (4a)$$

or with a good approximation for $T \sim T^0$

$$E_{21} = \frac{\Delta C_p \cdot T^0 \cdot (T^0 - T)}{2T} \qquad (4b)$$

with T^0 as the temperature where $E_{21} = 0$.
For calculating the temperature dependence of the two relaxation times according to eqn. 2, the nine parameters k^0_{12}, E_{12}, k^0_{21}, ΔC_p, T^0 for the folding reaction and k^0_{23}, E_{23}, k^0_{32}, E_{32} for the isomerization reaction have to be known.

2.2. *Comparison with two-state model*

Experimentally it is found that the fast relaxation time usually has only a very small relative amplitude [2,8]. The kinetics were, therefore, evaluated according to a two-state model:

$$N \underset{k_b}{\overset{k_f}{\rightleftharpoons}} U \qquad (5)$$

Assuming that D and D′ have the same optical properties allows to relate the two-state model to the isomerization model. The apparent two-state equilibrium constant K_a determined from optical measurements is then given by

$$K_a = (U)/(N) = k_b/k_f = k_{21}/k_{12}\,(1 + k_{23}/k_{32}) \qquad (6a)$$

and

$$1/\tau_2 = k_f + k_b \qquad (6b)$$

The apparent two-state rate constants k_f and k_b can in this way be calculated from the isomerization model. This allows a simple comparison between previous experimental results and the calculations. At the melting temperature T^m k_f equals k_b and a useful relation between different parameters is given by

$$\ln k^0_{21} = \ln k^m_{12} + \ln(1 + k^m_{23}/k^m_{32}) + E^m_{21}\,(1 - T^m/T^0)/RT^m \qquad (7)$$

with the superscript m referring to the values at T^m.

2.3. *Kinetic parameters for chymotrypsinogen A at pH = 2.0*

Calculations with different sets of parameters show that estimates for all of them can be obtained under favourable circumstances from published experimental data.

(a) For jumps from unfolding conditions ($K_a > 1$) to native ($K_a \ll 1$) the fast relaxation time will be mainly determined by k_{21} (and k_{23}), while the slow relaxation time is largely governed by k_{32}.

A fast relaxation time with a small amplitude was observed for chymotrypsinogen in the time range of 10–50 msec [8].

The slow relaxation time is of the order of 100 sec [2]. From the temperature dependence of τ_2 the activation energy E_{32} can be estimated. Model experiments on the cis–trans isomerization of proline residues show furthermore that the activation energy is the same for both directions within experimental error [1]. Therefore, one expects $E_{23} = E_{32} \simeq 20$ kcal/mole.

The ratio of the amplitudes of the fast to the slow relaxation curve under these conditions will be close to $D/D' = k_{32}/k_{23}$. A lower limit of $k_{23} \geqslant 6k_{32}$ is estimated for chymotrypsinogen. [2,8]

(b) For jumps to unfolding conditions where $K_a > 1$, there will usually be a region with $1/\tau_2 \simeq k_{12} \simeq k_f$ and a corresponding temperature dependence of $d(\ln \tau_2)/d(1/T) \simeq E_{12}/R$, allowing an estimate of k_{12} and E_{12}. But notice that at $K_a \gg 1$ the slow relaxation may be determined by k_{23} and the fast one by k_{12}.

(c) Calorimetric measurements give the change of enthalpy ΔH and of the specific heat ΔC_p upon unfolding of the protein [6,7]. Thus ΔC_p and, from the temperature where E_{12} equals ΔH, the reference temperature T^0 are obtained. From optical measurements T^m can be determined.

Together with eqn. 7 an estimate of all nine parameters is possible from experimental data. Fig.1 shows the kinetic data for the unfolding of bovine chymotrypsinogen A at pH 2.0, together with the calculated curves for τ_1, τ_2, k_f and k_b, using the

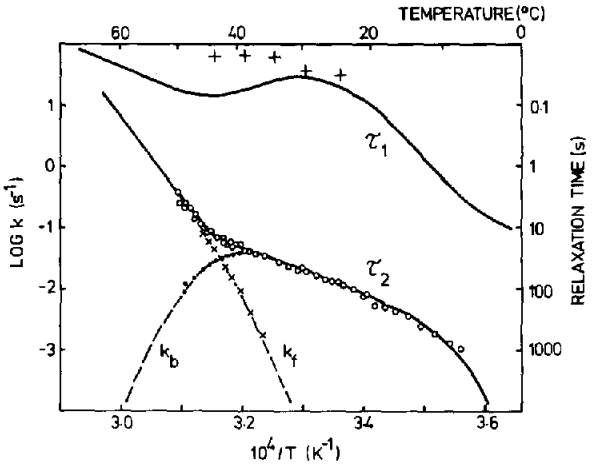

Fig.1. Temperature dependence of the kinetics of reversible unfolding of bovine chymotrypsinogen A at pH 2.0. (+) Fast relaxation time in $HClO_4$ solution, measured at 293 nm after temperature jumps of 5°C [8]. (○) Slow relaxation time measured after heating or cooling jumps of different size at 293 nm [2–4]. (×) Unfolding rate constant k_f and (•) refolding rate constant k_b, obtained by using a two-state model in their evaluation (N ⇌ U). Solid curves give the calculated temperature dependence using a minimal isomerization model (N ⇌ D ⇌ D') and the parameters of table 1.

formalism developed above and the parameters given in table 1.

3. Discussion

The minimal isomerization model leads to a very satisfying description of the equilibrium and kinetic data obtained on the reversible unfolding of globular proteins. Compared with the two-state model it resolves the pecularities in the temperature dependence

Table 1
Kinetic parameters for the unfolding of bovine chymotrypsinogen A at pH = 2.0 according to the minimal isomerization model (see text)

$k^0_{12} = 0.83 \times 10^{-3}\ s^{-1}$	$E_{12}/R = 30 \times 10^3$
$k^0_{21} = 26\ s^{-1}$	$\Delta C_p/R = 1.8 \times 10^3$
$k^0_{23} = 2.5 \times s^{-1}$	$E_{23}/R = 9.5 \times 10^3$
$k^0_{32} = 0.25 \times 10^{-3}\ s^{-1}$	$E_{32}/R = 9.5 \times 10^3$
$T^0 = 303$ K	

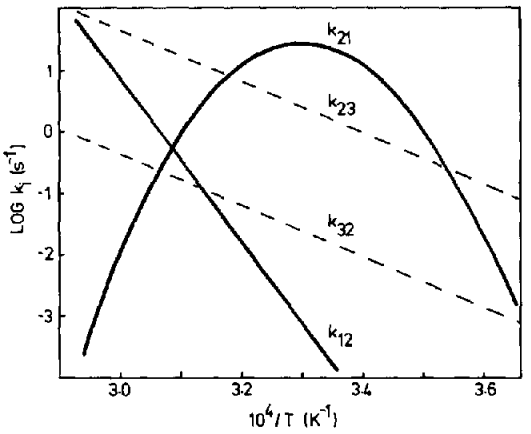

Fig.2. Temperature dependence of the overall rate constants for the unfolding of chymotrypsinogen A at pH = 2.0 according to the minimal isomerization model. Solid lines: Rate constants for unfolding k_{12} and refolding k_{21}. Broken lines: Rate constants for the isomerization of peptide bonds in the unfolded protein k_{23} and k_{32}.

of k_f and k_b, which had to be postulated in order to obtain agreement with equilibrium data.

Fig.2 shows the temperature dependence of the four overall or 'steady state' rate constants for chymotrypsinogen A at pH 2.0, the least accurate one being k_{23}.

At 0°C, the lifetime of D and D' will be the same ($k_{21} = k_{32}$) and close to 1000 sec. Electrophoresis of rapidly cooled, unfolded chymotrypsinogen A at pH 2.0 revealed two unfolded species with similar lifetime, in accordance with the model (S. A. Hawley, personal communication).

The maximum rate constant of refolding, k^0_{21}, of chymotrypsinogen A occurs close to physiological temperatures, if all peptide bonds are in the native conformation. This appears also to be the case for the other proteins studied up to now. Whether this is accidental or of biological significance has to await further experiments.

Acknowledgements

I thank Dr S. A. Hawley for information prior to publication, the Deutsche Forschungsgemeinschaft (Po 155/1) and the Fonds der Chemischen Industrie for support.

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