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Sergey A. Potekhin

Evgueni Kovriguine
Marquette University, evgueni.kovriguine@marquette.edu

Folding under inequilibrium conditions as a possible reason for partial irreversibility of heat-denatured proteins: computer simulation study

Sergey A. Potekhin
Institute of Protein Research, Russian Academy of Sciences, 142292, Pushchino, Moscow Region, Russia
Institute for Protein Research, Osaka University, 3-2, Yamadaoka, Suita, Osaka 565, Japan

Evgenii L. Kovrigin
Institute of Protein Research, Russian Academy of Sciences, 142292, Pushchino, Moscow Region, Russia

Abstract
Using computer simulations we have studied possible effects of heating and cooling at different scan rates on unfolding and refolding of macromolecules. We have shown that even the simplest two-state reversible transition can behave irreversibly when an unfavorable combination of cooling rate, relaxation time and activation energy of refolding occurs. On the basis of this finding we suppose that apparent irreversibility of some proteins denatured by heat may result from slow relaxation on cooling rather than thermodynamic instability and/or irreversible alterations of the polypeptide chain. Using this kinetic reversible two-state model, we estimated the effects of the scan rate and kinetic parameters of the macromolecule on its unfolding–refolding process. A few recommendations are suggested on how to reach maximal possible recovery after denaturation if refolding appears to be under kinetic control.

Keywords

1. Introduction

It is known that under most conditions heat denaturation of most proteins is partially reversible or fully irreversible. The usual conclusion drawn from such experiments is that heat causes irreversible changes in the protein structure. It is believed that processes leading to irreversibility involve aggregation, improper disulfide bond formation, or covalent alterations 1, 2, 3. The simplest model that is consistent with these observations is reversible unfolding followed by an irreversible process

$$N \rightleftarrows U \rightarrow D$$

locking the unfolded protein in a state from which it cannot refold over the time of the experiment.

A three-state model in which the first step of unfolding is treated by equilibrium thermodynamics and the second, irreversible step, by kinetics has been investigated 4, 5, 6. The fully kinetic model in which each step, i.e. unfolding, refolding and irreversibility, is treated in terms of the process rate has been examined as well [7]. But so far, the influence of refolding and cooling rates on reversibility of proteins denatured by heat under inequilibrium conditions without any irreversible process has not been studied.

This problem is of interest because the refolding rate of most proteins decreases with decreasing the temperature at least in the low temperature range 8, 9, 10. Therefore refolding at a sufficiently low temperature can be very slow. The unfolded conformation of proteins can become metastable in the temperature region where refolding is thermodynamically favorable. There are many reports demonstrating that folding of proteins is under kinetic control 11, 12, 13, 14. This suggests that some proteins which in principle fold reversibly may exhibit partial or complete irreversibility.

Theoretical analysis is generally used to reveal and describe the effects which can be produced by a real system. In this work, using computer simulations we demonstrate an `irreversible behavior' of a fully reversible two-state transition and determine under which conditions this phenomenon can take place. Our estimates aim to help experimentalists to interpret more adequately the results obtained in protein denaturation studies.

2. Methods

The simplest kinetic model describing unfolding-refolding of proteins requires two states:

$$N \rightleftarrows k_- k_+ D$$
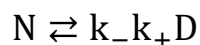
where N means the native species, D is the reversibly unfolded denatured form. We will assume that all the kinetic processes are of the first order and that the unfolding equilibrium constant is given by

$$K = [D] \, / \, [N] = k_+ \, / \, k_-$$

The temperature dependence of rate constants $k_\pm$ can be approximated by the Arrhenius relation in the form:

$$k_\pm(T) = 12_{\tau_d} \exp \Delta E_\pm R 1 T_d - 1T$$

where $\Delta E_\pm$ is the activation energy of unfolding (+) and refolding (−), R is the universal gas constant, T_d is the denaturation temperature (i.e. the temperature at which $K = 1$). It should be emphasized that all kinetic

processes for protein unfolding and refolding at a constant temperature can be described by simple exponential curves with the corresponding relaxation time τ. In our expressions τ_d is the relaxation time at the denaturation temperature:

$$\tau_d = [k_+(T_d) + k_-(T_d)]^{-1}$$

We also assume that neither the activation energy nor the unfolding enthalpy $\Delta H = \Delta E_+ - \Delta E_-$ depend on temperature. This assumption is not quite correct when studying conformational transitions in a wide temperature range. Nevertheless, it can be used as the first approximation to demonstrate the appearance of apparent irreversibility caused by a decrease in the renaturation rate. The applicability of this assumption for real proteins will be discussed elsewhere.

If the temperature changes with time according to the constant rate $V = |dT|/|dt|$, the fractional population of the unfolded state $\alpha = [D]/([N] + [D])$ is described by the differential equation (see Appendix A):

$$dadT = k_+ V \cdot 1 - aa_{eq},$$

where $\alpha_{eq} = K/(1 + K)$ is the fractional population of the unfolded state under equilibrium conditions (i.e. a sufficiently slow heating or cooling rate). Integration of this equation from the starting temperature T_0 gives the temperature dependence of the fractional population of the unfolded state:

$$a = \{a_0 + F(T)\}\exp[-J(T)],$$

where

$$J(T) = 1V\int T_0 T(k_+ + k_-)dT,$$

and

$$F(T) = 1V\int T_0 T k_+(T)\exp[J(T)]dT.$$

Numerical integrations were solved by standard methods for a range of k_+, k_- and V values.

3. Results and discussion

Our first interesting finding is a sharp qualitative distinction between protein unfolding (upon heating) and refolding (upon cooling) when the temperature changes rapidly. This distinction stems from the fact that both the unfolding and refolding rates in some cases increase with temperature, while the stability of the corresponding states changes in a different manner. The unfolded state (D) becomes more favorable while the initial state (N) becomes increasingly destabilized upon heating. There is clear asymmetry in the unfolding-refolding process with respect to the temperature. The result is that the protein entirely unfolds on heating, but the completeness of its refolding on cooling depends on the relationship between the relaxation time and scan rate. Fig. 1 illustrates the effect of the heating rate on the unfolding transition. The numbers alongside the transitions show the heating rate in K/min. The mark `eq' labels the fractional population α of the unfolded state upon very slow heating when chemical equilibrium between the native and denatured states is maintained. The curves α vs. T are shifted to a higher temperature on increasing the heating rate. It can be shown that an essential distortion of the equilibrium transition profile appears when the heating rate surpasses a certain value V_{max} as shown by the following expression (see Appendix B):

$$V > V_{max} = RT^2_d \Delta H \tau_d = \Delta T_{1/2} 4 \tau_d,$$

Where

$$\Delta T_{1/2} \approx 4RT^2_d \Delta H$$

is the half-width of the transition calculated according to the van't Hoff equation [2]. Below V_{max} the profile does not significantly depend on the heating rate and the unfolding can be regarded as equilibrium.
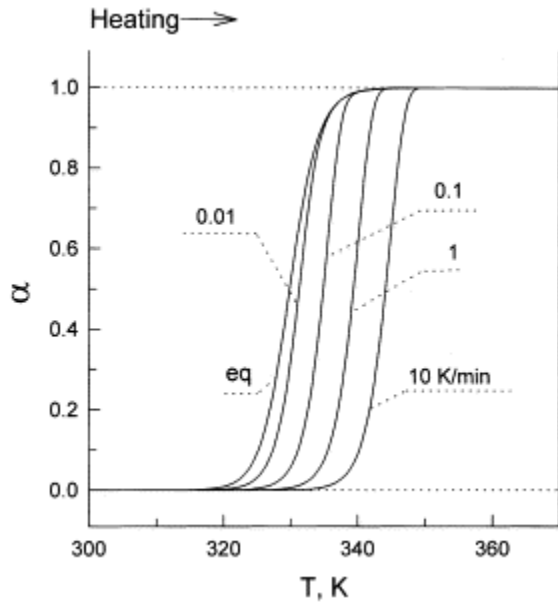

Fig. 1. Unfolding at different heating rates: the calculated denatured population α of the typical small globular protein vs. temperature (the values of scan rates are given in the figure; the mark `eq' implies an infinitely low scan rate). Parameters used in calculations are: $T_d = 330$ K, the midpoint of equilibrium transition; ΔH=100 kcal/mol, the enthalpy of transition; $\Delta E_+ = 110$ kcal/mol, the unfolding activation energy; $\tau_d = 10\,000\ s$, the relaxation time.

Using this result, we can estimate the conditions for a typical small globular protein (e.g. lysozyme) where the distortion of the transition profile becomes significant. Let us choose $\Delta H = 100$ kcal/mol, $\Delta E_+ = 110$ kcal/mol, $T_d = 330\ K$, $V = 1$ K/min [15]. In this case, heat denaturation will take place close to equilibrium only if the protein relaxation time does not exceed 130 s at $T = T_d$. However, it is known that the relaxation time for some proteins reaches dozens of minutes or even several hours [16], [17], [18], [19], [20]. Hence for such proteins both equilibrium unfolding and refolding is practically unattainable. In this case the shift of the apparent transition temperature T_{app} from T_d (an equilibrium denaturation temperature) is mainly determined by the heating rate, the activation energy and the relaxation time of the unfolding transition:

$$T_{app} \approx T_d + \Delta T_{1/2,kin} e1 + \ln 2 V \tau_d \Delta T_{1/2,kin}$$

where $\Delta T_{1/2,kin}$ is the half width of the transition at a particular heating rate (see Appendix C). Therefore at temperature T_{app} the denaturation rate constant is determined mainly by the heating rate, and the denaturation process will be indistinguishable from a one-step irreversible process [7], [21]. For some proteins studied by the scanning microcalorimetry technique, the heat denaturation is well described by a one-step kinetic model [3], [22]. In this case at the apparent temperature of denaturation, the rate constant can be determined by the formula [7]:

$$k + \left(T_{app}\right) = V \cdot \Delta E_+ R \cdot T^2_{app}$$

Since the deviations of the ratio $|\Delta E_+|/|T_{app}^2|$ caused by the protein and experimental conditions are usually not great (no more than 60% from the average), one can readily determine the constant of the rate at which protein denaturation takes place. If $||\Delta E_+|/|T_{app}^2| \sim 10^{-3}$ kcal mol^{-1} K^{-2} , the rate constant will be $\sim 8 \times 10^{-4}$ s^{-1} at the heating rate 0.1 K min^{-1}, which corresponds to a relaxation time of about 21 min. Taking into account that at the given parameters the rate constant changes by e (e is the base of natural logarithm) if the temperature changes by only 2°, it becomes clear that at the thermodynamic denaturation temperature T_d the relaxation time may be at least several hours.

In practice we cannot obtain the true denaturation temperature T_d from melting experiments if the relaxation time is sufficiently long to result in melting conditions which are obviously inequilibrium. If in this case the temperature drops some degrees below T_{app}, the protein should not necessarily refold because the real denaturation temperature might be significantly lower. A further decrease of the temperature will lead to a situation where the refolded state becomes thermodynamically favorable ($K < 1$). However, due to the slow relaxation the refolding rate at this temperature may become too small for the protein to refold within the time of the experiment.

Fig. 2 shows the effect of the cooling rate on the fractional population of the unfolded state. Notice that the rate of the temperature change affects the transition profiles very differently upon heating and cooling (cf. Fig. 1, Fig. 2). On heating the apparent temperature of transition will move upward along the temperature axis and the protein will necessarily unfold entirely because the unfolding rate increases with temperature. On the contrary, the apparent temperature of refolding upon cooling remains practically constant. Notice the most striking result: reversibility of the protein depends very much on the cooling rate. The protein cannot refold entirely if the sample temperature decreases rapidly, since the protein is not exposed to the optimal temperature for a sufficient time. At a low temperature, refolding can slow down so that the protein is not able to return to its native conformation. If the relaxation time is shorter, the protein will be able to refold during the available time at a cooling regime. However, even in this case inequilibrium during cooling always increases the life time of proteins in the unfolded state and thus enhances irreversible processes (e.g. aggregation) of the unfolded protein chains.
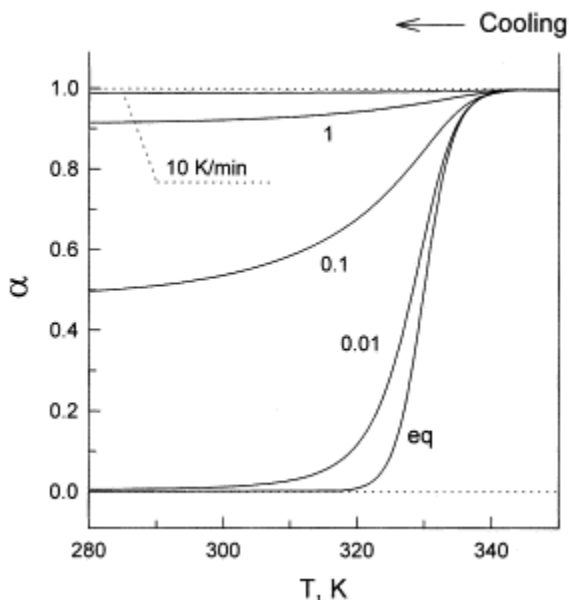
Fig. 2. Refolding at different cooling rates: the calculated denatured population α of the typical small globular protein vs. temperature (the values of scan rates are given in the figure; the mark `eq′` implies an infinitely low scan rate). Parameters used in calculations are the same as in <u>Fig. 1</u>.

It is worth noting that, as a rule, partial or complete irreversibility is interpreted as a consequence of some additional irreversible process (e.g. <u>17</u>, <u>18</u>). Our results show that an assumption of such processes is not necessary to explain incomplete refolding. When the renaturation rate becomes sufficiently low at temperatures below the thermodynamic transition temperature T_d, the protein sample will remain partially unfolded in the experiments.

Notice that irreversibility observed in spectroscopic or calorimetric investigations of heat denaturation (having relatively fast rates of cooling between scans) should not be confused with that protein renaturation studies involving long incubation under native conditions. At optimal conditions proteins which exhibit partial irreversibility in experiments including fast cooling could be capable of complete refolding after long annealing at optimal temperature.

The dependence of the activation energy on temperature in real proteins can impose restrictions on the conclusions made in this paper. It was shown for some proteins that the activation energy of denaturation ΔE_+ changes only slightly with temperature. ΔC_p of activation of the denaturation process does not exceed 20% of the entire heat capacity increment upon denaturation <u>[8]</u>. The activation energy of refolding changes drastically and drops to zero in the temperature range 30–50°C. Hence, if k_+ increases practically in the whole temperature range, k_- is maximal at the temperature where ΔE_- is zero.

Strictly speaking, the presented conclusions are applicable only in cases when the renaturation rate increases with increasing temperature and the real temperature of denaturation is below the temperature of the maximal renaturation rate. On the other hand, the real denaturation temperature cannot be lower than the temperature of the maximal protein stability, i.e. the temperature at which the denaturation enthalpy is zero <u>[2]</u>. Thus the phenomenon of incomplete protein renaturation as a result of a low folding rate and the dependence of renaturation on the cooling rate can be observed only if the denaturation temperature T_d is between the temperature of maximal stability ($\Delta H = 0$) and the temperature of maximal renaturation rate ($\Delta E_+ = 0$). Probably Tani et al. <u>[20]</u> observed a similar phenomenon when studying refolding of heat-denatured ovalbumin.

It is also possible that protein would not renature upon cooling in any other case if the renaturation rate (even at the temperature of its maximal value) is rather low for the real folding to occur.

Another case where the described dependence of the extent of the transition would undoubtedly be observed is cold denaturation of proteins <u>[23]</u>. The rate of cold denaturation does increase with temperature because $T_{d,cold}$ is lower than the temperature of maximal stability. Therefore provided its value is low enough, denaturation upon cooling would be incomplete or would not occur at all.

On the basis of our considerations we can define the optimal condition for refolding: the temperature T_{opt} which is below the true denaturation temperature and thus represents conditions where the equilibrium constant is much less than unity ($K \ll 1$) but the refolding rate is still moderate. Let us estimate the optimal temperature for 90% maximal recovery (when $K(T_{\text{opt}}) = 0.1$). Taking into consideration that

$$K(T_{\text{opt}}) = \exp - \Delta G(T_{\text{opt}})RT_{\text{opt}} \approx \exp \Delta H(T_{\text{opt}} - T_d)RT^2{}_d$$

we arrive at $T_{\text{opt}} \approx T_d - 0.6\Delta T_{1/2}$.

For small globular proteins ($T_d = 330$ K, $\Delta H = 100$ kcal/mol) we estimated $\Delta T_{1/2} \approx 9$ K. Thus, the optimal temperature for refolding of small globular proteins should be about 5–6° below the true denaturation temperature.

On the basis of the above results we can try to formulate a few recommendations on how to choose optimal conditions for refolding of an `irreversibly' unfolded protein if its `irreversibility' is suggested to be kinetic. To do so, one should:

1. have a melting curve for the protein at a heating rate as slow as possible to get the upper limit of T_{app} for the true denaturation temperature;
2. evaluate the relaxation time and maximal population of the native state at T_{app} using the temperature jump or any condition jump experiments (to bring the protein from its native state to the conditions under study);
3. while in a temperature jump experiment, estimate the maximal temperature at which the protein does not yet denature. The value of this temperature may be governed by a small equilibrium constant or a high relaxation time. These two effects are easily separated if with the temperature jump data. If the relaxation time does not grow fast enough on decreasing the temperature, there is a chance to identify the temperature region in which the native state is reasonably populated and the refolding rate is still not too small to allow the protein to reach its equilibrium level of the native population during a reasonable time. Thus the optimal conditions for refolding are a compromise between thermodynamic stability of the native species and its relaxation time.

4. Conclusions

In this work we showed by computer modelling that the experimentally observed thermal irreversibility of some proteins does not always reflect actual irreversibility. We found that a fully reversible two-state transition can appear partly (or fully) irreversible when an unfavorable combination of the transition parameters (kinetic and equilibrium) and the scan rate is achieved. This phenomenon will be observed when the activation energy of refolding is positive.

After heat denaturation, the protein sample is cooled at a certain rate and in routine experiments the scan rate is rarely lower than 5 K/h. However, even such a slow cooling can be too fast if the relaxation time of the protein refolding process exceeds a certain value. As a result, the protein sample can reach room temperature being not completely refolded with its unfolded species kinetically trapped.

We analyzed both analytically and numerically the two-state reversible transition model and determined the conditions under which this `kinetic' irreversibility can appear.

Acknowledgements

Appendix A. The kinetic equation

$$N \rightleftarrows k_- k_+ D$$
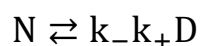
where N is the native species, D is the reversibly denatured form, in terms of rates can be described by the differential equation:

$$d[N]dt = d[D]dt = -k_+[N] + k_-[D] \qquad \text{(A1)}$$

where d/dt is a derivative with respect to time.

Taking into consideration that the fractional population of the denatured form is

$$a = [D][N] + [D] \qquad \text{(A2)}$$

and dividing Eq. (A1)by ([N]+[D]), it can be easily deduced that

$$dadt = k_+(1 - a) - k_-a = k_+[1 - (1 + k_+/k_-)] \qquad \text{(A3)}$$

Since the equilibrium constant $K=k_+/k_-$ and the equilibrium fractional population of the unfolded state is

$$a_{eq} = K1 + K \qquad \text{(A4)}$$

Eq. (A3)can be transformed into the following form

$$dadt = k_{+1} - aa_{eq} \qquad \text{(A5)}$$

We will assume that temperature changes with time according to the constant scanning rate $V = |dT|/|dt|$. Thus, the relevant differential equation will be:

$$dadT = k_+V1 - aa_{eq} \qquad \text{(A6)}$$

Appendix B.

It is easy to see that $V\tau = \Delta T_{cor}$ has the meaning of the correlation temperature domain. In other words, it shows how far along the temperature the system remembers its previous state on heating. The maximal change of equilibrium population of unfolded state over this interval can be evaluated as

$$\Delta a_{eq} \approx \Delta T_{cor} da_{eq} dT$$

Taking into consideration that the maximal deviation of α from α_{eq} cannot surpass the maximal change of equilibrium population over the correlation temperature domain $\Delta\alpha_{eq}$ and that at the temperature of denaturation

$$da_{eq}dT \approx C_{p,max}\Delta H \approx H4RT^2{}_d$$

it can be easily deduced that

$$\Delta a \leq \Delta a_{eq} \approx \Delta HV\tau_d 4RT^2{}_d$$

Finally, we assume that if we wish to heat as fast as possible but still wish to have a close-to-equilibrium melting profile, the difference between `equilibrium' and `kinetic' populations of the unfolded state should not exceed 0.25. Thus, for the maximal `equilibrium' scan rate V_{max} we have

Appendix C.

In the case of $V > V_{max}$ the apparent transition temperature starts to deviate from T_d and finally coincides with the value of T_{app} for irreversible denaturation with the same rate constant [7]. Taking into consideration the Arrhenius relation for the rate constant k_+ (see `Theory') and that for irreversible denaturation

$$k_+ = V \Delta E_+ R T^2{}_{app},$$

we obtain

$$2\tau_d V \Delta E_+ R T^2{}_{app} = \exp \Delta E_+ R 1 T_d - 1 T_{app}$$

or

$$\ln 2 \tau_d V \Delta E_+ R T^2{}_{app} = \Delta E_+ R T_{app} - T_d T_{app} T_d.$$

In the case of irreversible denaturation, the parameters of the excess heat capacity relate to the activation energy as follows [20]:

$$\Delta E_+ = e R T^2{}_{app} C_{p,max} \Delta H$$

Taking into consideration that $\Delta T_{1/2} \approx C_{p,max}/\Delta H$ we obtain

$$T_{app} \approx T_d + \Delta T_{1/2,kin} e 1 + \ln 2 V \tau_d \Delta T_{1/2,kin}$$

where $\Delta T_{1/2,kin}$ is a half width of the transition at the particular heating rate.

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