Pathways in Two-State Protein Folding

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ABSTRACT Thermodynamic measurements of proteins indicate that the folding to the native state takes place either through stable intermediates or through a two-state process without intermediates. The rather short folding times of proteins indicate that folding is guided through some sequence of contact bindings. We discuss the possibility of reconciling a two-state folding event with a sequential folding process in a schematic model of protein folding. We propose a new dynamical transition temperature that is lower than the temperature at which proteins in equilibrium unfold. This is in qualitative agreement with observations of in vivo protein folding activity quantified by chaperone concentration in *Escherichia coli*. Finally, we discuss our framework in connection with the unfolding of proteins at low temperatures.

INTRODUCTION

Proteins appear to fold into a unique native conformation, in spite of an astronomical number of alternative configurations. This apparent paradox, usually attributed to Levinthal (1968), is further sharpened in view of the fact that there is experimental evidence that the folding transition behave nearly like a two-state system for many single-domain proteins (Privalov and Khechinasvili, 1974; Creighton, 1992; Baldwin and Rose, 1999a,b). This means that for these proteins, the transition from denatured to native state occurs rather directly, without observed intermediates. One would think that such a two-state behavior would exclude the possibility of guiding the protein to the native state. The purpose of this paper is to quantify the degree of guiding that is compatible with the observed two-state folding process. We do this through generalizing a hierarchical protein model introduced earlier (Hansen et al., 1998). In this model we parameterize the folding process through an ordered series of binding events, and thereby obtain a first-order folding-unfolding process. However, as intermediates will be associated to guiding the folding, the original model does not give a two-state folding transition.

The folding of proteins can be addressed experimentally by thermodynamic quantities such as entropy, enthalpy (H), and heat capacity ($C = dH/dT$) as functions of temperature. One characterizes the folding transition with the released energy, i.e., the latent heat (Q), and the peak height of the heat capacity (ΔC) at the transition temperature T_c. T_c is defined as the temperature at which the protein has equal free energies in the native (folded) and denaturated (unfolded) states. The van't Hoff relation (Privalov, 1979),

$$\Delta H = \alpha k T_c^2 \frac{\Delta C}{Q}, \tag{1}$$

provides a powerful way to quantify the sharpness of a smoothed out first-order phase transition taking place at T_c. It relates the enthalpy difference between the two phases, ΔH, to the height of the heat capacity peak, ΔC, and latent heat of the transition, Q, which is the same as ΔH, i.e., $\Delta H = Q$. α is a dimensionless proportionality factor and k is the Boltzmann constant. For a given ΔH and Q, then, the value of α is inversely proportional to ΔC; in this respect, a smaller α corresponds to a sharper transition.

When the transition is two-state it is known that $\alpha = 4$ (Privalov, 1979). We will also show that when the transition has a large number of equally stable intermediates, then $\alpha = 12$. For the single-domain proteins, ribonuclease, lysozyme, chymotrypsin, cytochrome c, and myoglobin, Privalov and Khechinasvili (1974) find experimentally

$$\alpha = 4.2 \tag{2}$$

to within 5% accuracy, demonstrating that these transitions are very nearly two-state.

Protein folding can be described on a number of different levels. On a microscopic level it is governed by molecular forces between amino acids and between amino acids and the surrounding water. On a large scale one may characterize the folding by a number of binding events that each limits the residual conformational entropy. The ordering of these binding events is at present unknown, although recent experimental studies suggest some sort of hierarchical ordering in the folding process (Baldwin and Rose, 1999a,b; Nolting et al., 1997; Chakraborty and Pang, 2000). This may contrast somewhat to protein folding as a two-state process. In this paper we explore the the possibility for reconciling a two-state thermodynamics with a guided folding process. As a simple guiding principle, we adopt the sequential zipper-like (Schellman, 1958; Dill et al., 1993)

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description of the process (Hansen et al., 1998). In contrast to geometrical zipper models implemented for, e.g., DNA melting, we here can also view the zipper as an effective description of a unique folding pathway, i.e., an hierarchical ordered sequence of binding events between different parts of the protein (Hansen et al., 1998).

THE MODEL

We sketch the model and its parameterization in the following. One may visualize each binding event as closing of a specific pair contact between two residues. Each of these events is characterized by binary variable ψ_i that indicates whether it is closed ($\psi_i = 1$) or open ($\psi_i = 0$). The overall folding state of the protein is thus characterized by the set of binary variables $\psi_1, \psi_2, \ldots \psi_N$, where the native state is the one where all $\psi_i = 1$. There is experimental evidence that protein folding happens through a fairly specific pathway, i.e., that there is an ordering of binding events leading to the native state (Nolting et al., 1997; Chakraborty and Pang, 2000; Huang et al., 1999). Mathematically, the existence of a specific pathway is implemented by the series of inequalities

$$\psi_i \geq \psi_{i+1}\,. \tag{3}$$

The variables ψ_i are insufficient to describe the degrees of freedom for the protein. In order to take these into account, we introduce a second independent set of variables, ξ_i, which describes the degrees of freedom associated with the unfolded parts of the protein. In principle, these will have a range of possible values, analogous to the about various possible values of the dihedral angles of the protein (Creighton, 1993). However, for simplicity, we then assign only two values, ϵ_0 or $\epsilon_0 - E$, to each ξ_i. The Hamiltonian is

$$\mathcal{H} = -\sum_{i=1}^{N} \psi_i \xi_i\,, \tag{4}$$

with the constraints in Eq. 3 incorporated on the ψ_i values, implying that when $\psi_i = 0$ all terms with $j \geq i$ possess no energy. The interpretation of the terms in this Hamiltonian is that when a local binding is intact, $\psi_i = 1$, there is an energy cost of E to change the ξ_i variable from the value $-\epsilon_0$ to $-\epsilon_0 + E$. When there is no binding, that is, $\psi_i = 0$, there is no energy cost associated with changing ξ_i; it "flaps" freely. We stress that we have simplified the conformation space here to only two states, with energy $-\epsilon_0$ and $-\epsilon_0 + E$, per variable ξ_i of the polypeptide. In reality already the individual amino acids will have more dihedral angles to choose from, and the true energy spectra will presumably have one lowest energy state and a number of higher energy states that become accessible when the structure flaps freely.

We note that for any finite value of E, the protein may change structure locally due to change in ξ_i even in the parts of the protein where $\psi_i = 1$. This would then reflect an unfolding event inside a protein. In order to simplify the analysis we assume E to be sufficiently large compared to any other energy scale in the system—in particular T, where T is the temperature—so that the ξ_i variables never take the value $\epsilon_0 - E$ when $\psi_i = 1$. Hence, in our model no unfolding can occur inside an already folded part of the protein. We have put the Boltzmann constant equal to unity or absorbed it into the temperature for simplicity.

We may define a set of binary, unconstrained variables φ_i, taking the values 0 or 1 such that

$$\psi_i = \varphi_1 \cdots \varphi_i\,. \tag{5}$$

In particular, $\psi_1 = \varphi_1$. In the limit when $E \to \infty$, the Hamiltonian (4) becomes

$$\mathcal{H}_{p1} = -\epsilon_0(\varphi_1 + \varphi_1\varphi_2 + \varphi_1\varphi_2\varphi_3 + \cdots + \varphi_1\varphi_2 \cdots \varphi_N)\,, \tag{6}$$

where there are no additional constraints. The role of the variables ξ_i is now played by the degeneracy present in Eq. 6, as one $\varphi_i = 0$ implies that $\mathcal{H}_{p1}$ is independent of all subsequent φ_j variables ($j > i$). If i labels the first variable where $\varphi_i = 0$, then $\mathcal{H}_{p1} = -(i-1)\epsilon_0$, and the number of degenerate states at this energy is 2^{N-i}, reflecting the residual degrees of freedom. This is because variable i is open, and the rest of the $N - i$ variables access two possible degenerate states according to the previous discussion about the dihedral angles. This allows an exact calculation of the partition sum of the system, by summing over the number i where first $\varphi_i = 0$:

$$Z = \sum_{i=1}^{N} 2^{N-i} e^{\beta\epsilon_0(i-1)} + e^{\beta N\epsilon_0} = 2^{N-1}\frac{1 - e^{N(\beta\epsilon_0 - \ln 2)}}{1 - e^{\beta\epsilon_0 - \ln 2}} + e^{\beta N\epsilon_0} \tag{7}$$

which rapidly changes or have a smoothed out singularity at $\beta = 1/T = \ln 2/\epsilon_0$ corresponding to a first order transition at $T_c = \epsilon_0/\ln 2$.

We stress that the number 2 in the above degeneracy count is an artifact of assuming that each variable has only two possible states in the unfolded state. The real degeneracy count can have a different degeneracy factor.

At the transition the ordered, fully folded state $\{\varphi_i\} = \{1111\cdots1\}$ has an energy $U = -\partial \ln Z/\partial\beta = -N\epsilon_0$. Thus $\Delta H = Q = N\epsilon_0$ and $\Delta C = \partial U/\partial T = N^2(\ln 2)^2/12$ (at T_c) unfolds to a disordered structure with energy $U = 0$, leading to $\alpha = 12$ by use of Eq. 1. This corresponds to a situation where there are many intermediate states of the same free energy. This will smooth out the transition and result in a broader peak in the heat capacity. On the other hand, we may consider only a rescaled last term

$$\mathcal{H}_{p2} = -N\epsilon_0\varphi_1\varphi_2 \cdots \varphi_N\,, \tag{8}$$

such that the partition function becomes $Z = 2^N - 1 + e^{\beta N\epsilon_0}$. Then one also obtains a sharp phase transition at $T_c = \epsilon_0/\ln 2$, with $\Delta H = Q = N\epsilon_0$ but with $\Delta C = (N\epsilon_0 \ln 2)^2/4$. Using Eq. 1, this will lead to a value $\alpha = 4$, as expected because this is a description of a classical two-state system (Privalov and Khechinasvili, 1974). The Hamiltonian in Eq. 8 describes a situation in which the system only lowers energy when all contacts are closed, and meaning that the protein is in the unique native state.

There is no guiding in the Hamiltonian in Eq. 8, since the ground state, $\{1111\cdots111\}$, is one out of the 2^N possible states, whereas all the other $2^N - 1$ states are degenerate. Thus the time to find the ground state for such a two-state system will be very long when simulating by Monte Carlo, as we will now discuss.

RESULTS AND DISCUSSION

We define time in the model based on the Monte Carlo Metropolis (MC) method (Binder, 1987). The values of φ_i are chosen or changed randomly, and acceptance of each choice depends upon the usual Boltzmann factor due to any energy shift connected to this. Time advances by one unit for every attempted update of one of the φ_i variables. We note that in principle the dynamics of an MC procedure is different from the actual dynamics of a given Hamiltonian, although properties at thermal equilibrium are properly represented. However, if time scales associated to different φ_i variables are not too different from each other, the MC simulation may reflect the overall dynamical behavior.

We measure the average folding time as the typical number of states visited before finding the ground state. This time is widely different between the guided in Eq. 6, and the two-state model in Eq. 8. For the true two-state model the average folding time is $2^N/2$. This is because no variable will be fixed at 1 before all variables are 1, thus making a probability of $1/2^N$ of reaching the ground state at each time step, irrespective of what the previous state was. Thus, the two-state system indeed takes exponential times to fold, thus confirming the Levinthal paradox of astronomical folding times for unguided protein folding.

For the guided system governed by Eq. 6, the ground state is found in a time growing as N^2, as in a diffusion process, when T is below T_c. This reflects that at each time step only one variable can be fixed at the value $\varphi_i = 1$, the one where the previous vaiable equals 1 (i.e., $\varphi_{i-1} = 1$). Attempts to change other variables will either be energetically disfavored (for $j < i$) or likely be subjected to reversals at later stages because these conformational changes are not associated with any energy changes. When each time step allows one variable to possibly change value, it typically takes N time step to fix the next φ on the pathway. Summed over all subsequent variables, this gives an overall folding time scaling as N^2. The exact prefactor to this folding time depends on temperature, as increased temperature enhance the probability that an already folded variable unfolds ($1 \rightarrow 0$) again.

To reconcile that a large class of proteins behaves as a two-state system with the necessity of being able to reach the ground state in a reasonable time, we now study a combination of the two Hamiltonians in Eqs. 1 and 4:

$$\mathcal{H}_p = \lambda_p \mathcal{H}_{p1} + (1 - \lambda_p)\mathcal{H}_{p2}\,. \tag{9}$$

$\lambda_p \in [0, 1]$, is a dimensionless parameter that weighs the contributions from the Hamiltonians $\mathcal{H}_{p1}$ and $\mathcal{H}_{p2}$. This Hamiltonian has a transition at $T_c = \lambda_p\epsilon_0/\ln 2$ as shown below. We can define a partial free energy $F(n)$, where $n + 1 = i$ according to i in Eq. 1. Consequently, $F(n)$ is nth term in the partition sum ($n\epsilon\{0..N\}$). The partition function becomes

$$\begin{aligned} Z = e^{-\beta F} &\equiv \sum_{n=0}^{N} e^{-\beta F(n)} \\ &= \sum_{n=0}^{N-1} e^{-\beta[n(T\ln 2 - \lambda_p\epsilon_0) - T(N-1)\ln 2]} + e^{\beta N\epsilon_0} \\ &= 2^{N-1}\frac{1 - e^{N(\beta\lambda_p\epsilon_0 - \ln 2)}}{1 - e^{\beta\lambda_p\epsilon_0 - \ln 2}} + e^{\beta N\epsilon_0} \end{aligned} \tag{10}$$

For a given temperature the partial free energy of states is $F(n \le N - 1) = n(T\ln 2 - \lambda_p\epsilon_0) - T(N - 1)\ln 2$, and $F(N) = -N\epsilon_0$.

In Fig. 1 we show $F(n)$ schematically for different temperatures T, where we set $\epsilon_0 = 1$ here and in the following discussion. Each $F(n)$ exhibits a jump at $n = N$ corresponding to the free energy gain $N(1 - \lambda_p) + \lambda_p$ for reaching the ground state. At low T, $F(n)$ is monotonically decreasing, reflecting a fast folding kinetics where the typical folding

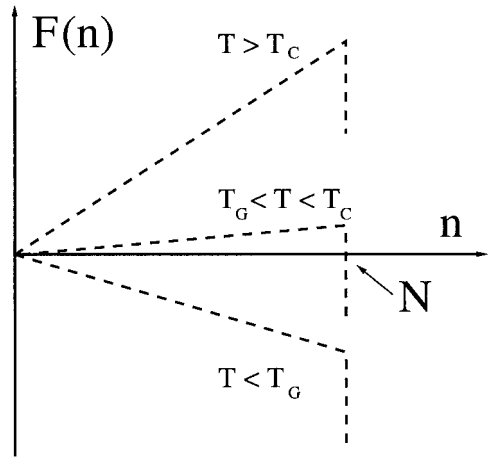

FIGURE 1 A schematic drawing of the partial Gibbs free energy F(n) defined through Eq. 1 as a function of the level of folding n for for three different temperatures T. $F(0)$ is rescaled to 0.

time grows as N^2. At an intermediate $T = T_G = \lambda_p/\ln 2$ all $n < N$ are equally probable. Below this temperature guiding becomes important. Also, T_G is lower than the folding-unfolding transition temperature T_c where the denaturated state becomes thermodynamically favored. For T in the interval between T_G and T_c the intermediate states are unstable (see Fig. 1)—i.e., they form a barrier between the folded and denatured state—and the folding time scale exponentially with both T and N. At a higher $T = T_c = 1/\ln 2$ the folded state becomes unstable, and the protein unfolds ($\langle n \rangle \approx 0$). The fact that the free energy landscape changes with T means effectively that two-state folding around T_c is compatible with guiding and fast folding at low T.

Fig. 2 shows the van't Hoff coefficient α as a function of λ_p on the unit interval based on direct calculation of the partition function. One observes that increasing λ_p—i.e., increasing the guiding—leads to increasing α and thus to a softening of the transition. As N is increased, the regime where α is very close to 4 is expanded toward higher values of λ_p. For example, with the experimental observation of $\alpha = 4.2$, and assuming $N = 10$, λ_p is close to zero, whereas for $N = 100$, λ_p is approximately 0.7. Thus, in this latter case, 70% of the energy difference between the unfolded and folded states sits in the guiding, i.e., comes from $\lambda_p \mathcal{H}_{p1}$ and still α is very close to the value, indicating the folding process to be essentially a two-state process.

We now discuss the fact that large N allows for more guiding, i.e., larger λ_p, without destroying the two-state nature of the transition. To understand this we note that any $\lambda_p < 1$ in fact define a virtual phase transition at $T = T_G < T_c$. At T_G the protein would unfold if it were not due to the additional gain in binding energy when the ground state is reached. This virtual transition is not seen directly in equilibrium thermodynamics, but strongly influences the MC dynamic behavior in the temperature range between T_G and T_c. In this intermediate regime the protein is a two-state system due to the appearance of a free energy barrier (see Fig. 1). In order to cross this barrier, a large thermal fluctuation is needed. Such a fluctuation is rare and hence the folding time will be long. When the system finally is folded, it will stay so for $T < T_c$ if N is large enough, and as a consequence $\alpha = 4$ for the real transition. However, for systems with small N, it may unfold again due to thermal fluctuations that take it across the barrier in the opposite direction. Once out of the folded state, it will linger on the "wrong" side of the barrier, where it essentially only sees the Hamiltonian H_{p1}, which gives $\alpha = 12$.

Experimentally, if one is dependent on dynamics, one presumably measures T_G as the transition temperature, whereas for experiments based on thermodynamics it would be T_c. For fast living organisms such as *Escherichia coli* the overall status of fraction of unfolded proteins can be monitored by the level of chaperone DnaK (Alberts et al., 1994; Arnvig et al., 2000). By means of energy input from ATP, unfolded proteins are produced in vivo. In a living cell these are thermodynamically unstable and want to fold. The speed of the folding process is increased or catalyzed by chaperones. For temperatures between 13 and 37°C the DnaK per *E. coli* cell raises slowly from 4000 to 6000, whereafter it rises sharply to ~8500 at 42°C and ~18,000 at 46°C (Herendeen et al., 1979; Pedersen et al., 1978). At 50°C the *E. coli* dies. This may be taken as an indication that in the temperature interval above 37°C, the typical proteins need help in the folding process. But as the cell is able to sustain life up to about 50°C, the typical proteins must have some stability up to this higher temperature. This resembles the behavior of our model, with a T_G of about 37°C, an exponentially slow folding of proteins necessitating the help of chaperones at higher temperatures, and a T_c of the order of 50°C (Arnvig et al., 2000).

The above considerations can be extended to include a more realistic scenario in which the protein is reacting with

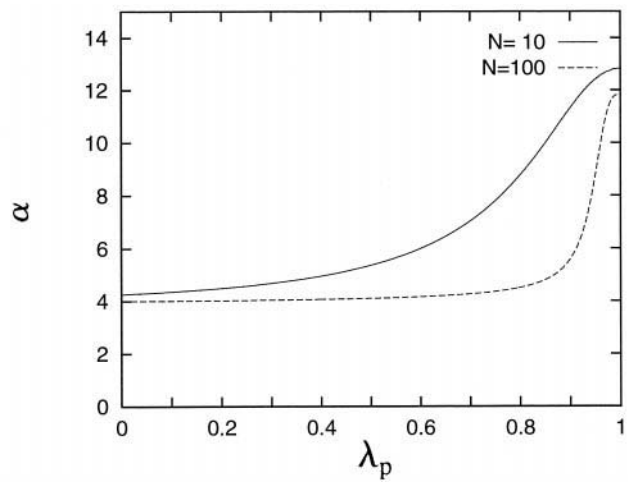

FIGURE 2 The van't Hoff coefficient α as a function of λ_p for $N = 10$ and 100.

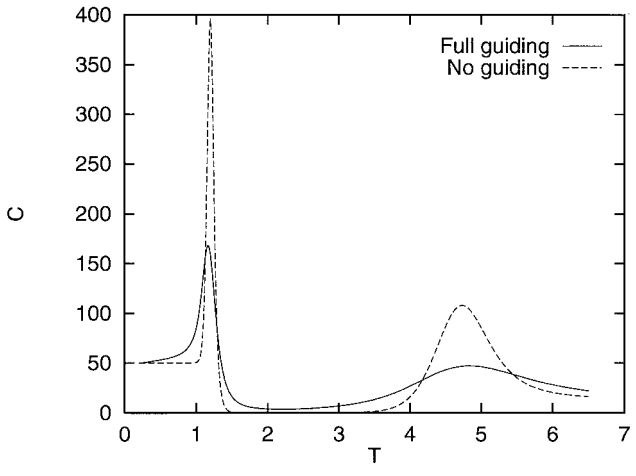

FIGURE 3 Heat capacity curves for a system $N = 50$ with and without guiding, i.e., with $\lambda_p = \lambda_w = 1$ respectively $\lambda_p = \lambda_w = 0$. The parameters for the water variables are $\epsilon_{min} = -3.1$, $\Delta = 0.04$, and $g = 350$.

water. Following Hansen et al. (1998), we parameterize this through water variables $w_1, w_2, \ldots, w_N$, taking values $\epsilon_{min} + s\Delta$, $s = 0, 1, \ldots, g - 1$. Here, Δ is the spacing of the energy levels of the water-protein interactions. We quantify the coupling to the water by a combination of the Hamiltonians

$$\mathcal{H}_{w1} = (1 - \varphi_1)w_1 + (1 - \varphi_1\varphi_2)w_2 + \cdots + (1 - \varphi_1\varphi_2 \cdots \varphi_N)w_N , \quad (11)$$

and

$$\mathcal{H}_{w2} = (1 - \varphi_1\varphi_2 \cdots \varphi_N)(w_1 + \cdots + w_N) , \quad (12)$$

to form the total Hamiltonian

$$\mathcal{H} = \lambda_p\mathcal{H}_{p1} + (1 - \lambda_p)\mathcal{H}_{p2} + \lambda_w\mathcal{H}_{w1} + (1 - \lambda_w)\mathcal{H}_{w2} . \quad (13)$$

The dimensionless parameter $\lambda_w \epsilon [0,1]$ measures the contributions from the Hamiltonians $\mathcal{H}_{w1}$ and $\mathcal{H}_{w2}$, while λ_p is the same parameter defined in Eq. 5. (Here it may be noted that $\mathcal{H}_{w2}$ will introduce non-local interactions between distant units, when the terms are interpreted using the variables ψ_i and ξ_i.) When $\lambda_p = \lambda_w = 1$ we are back to the Hamiltonian defined by Hansen et al. (1998) whereas when $\lambda_p = \lambda_w = 0$ we are facing a two-state Hamiltonian. In Fig. 3 we display the heat capacity curves for these two extremes. The system is folded in its ground state between the cold unfolding transition at $T = 1.2$ and the hot unfolding transition at $T = 4.7$. As also quantified by the van't Hoff coefficients, we see that the Hamiltonian without guiding gives a phase transition which is sharper by a factor of about 3 for both cold and hot unfolding transitions. Also, in terms of temperature, these transitions are much more separated than in real systems. The present model as it stands is not able to account for this.

In Fig. 4 we investigate systematically the van't Hoff

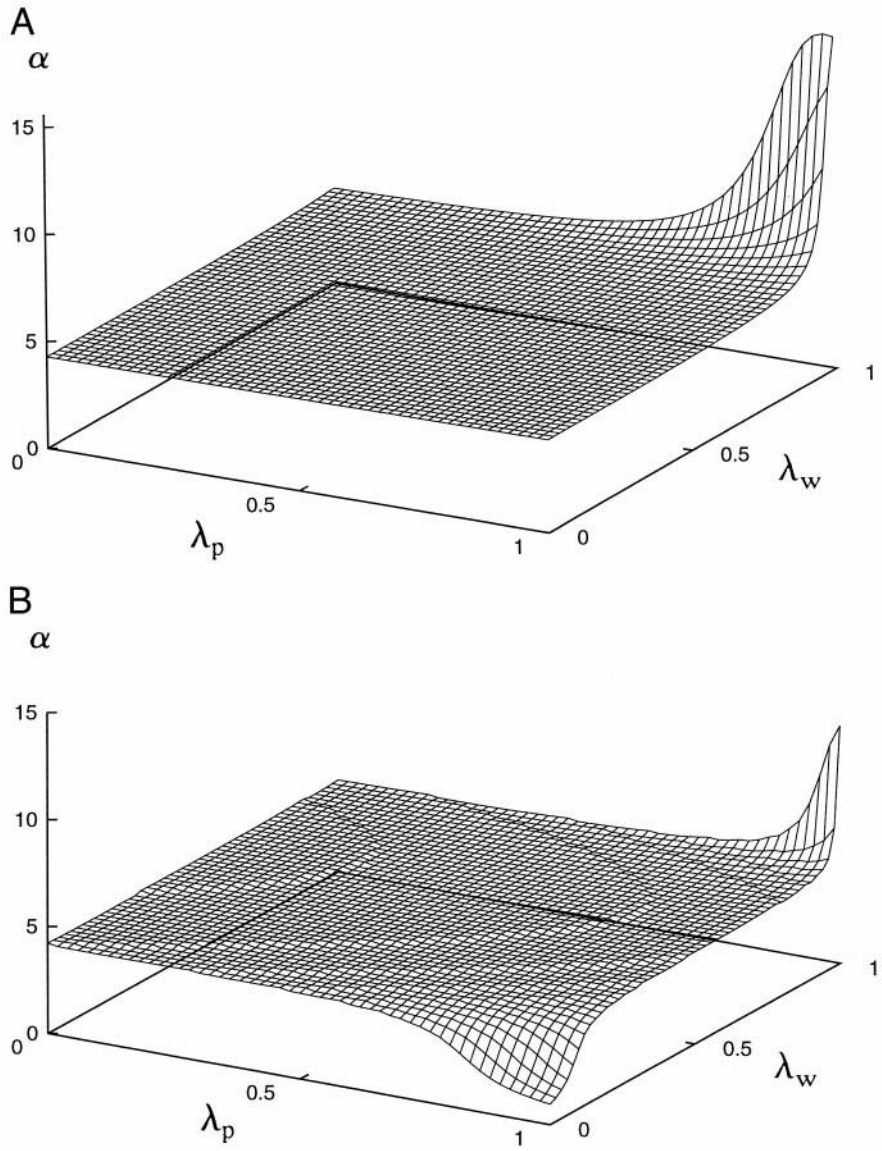

FIGURE 4 van't Hoff coefficient α for hot (*a*) and cold (*b*) transition for $N = 100$ system. The other parameters are as in Fig. 3.

coefficient α as function of λ_p and λ_w for the hot (Fig. 4 *a*) and the cold (Fig. 4 *b*) transition. As is evident, α is similar but somewhat larger for the hot than for the cold transition. As a consequence, the cold transition transition is slightly sharper. We are not aware of any explicit experimental measurements of the van't Hoff coefficient for the cold transition, but Privalov et al. (1986) indicate a sharp unfolding of metmyoglobin at the the cold transition. Such a measurement will in practice be hampered as the cold transition is mainly seen experimentally at pH values where it is close to the hot transition.

Finally, we note the distinct feature of the cold transition α when $(\lambda_p,\lambda_w) \approx (1,0)$ where it drops to a value below 4. This artifact incidently is due to a merging of two neighboring cold transitions, as it can be shown that α can not be smaller than 4 for a single transition.

We summarize by noting that in this protein model, it is easy to reconcile the thermodynamics of a two-state system with the dynamics of a guided system, as this can be done by diminishing λ_p and/or λ_w from the value one. The dynamical consequence of the hereby masked guiding is a folding time that is dramatically reduced when the temperature is moving below the transition temperature.

We note as final consequence of our model that good folders can be viewed as random sequences of folding steps of which the last steps have a particularly favorable binding energy thereby securing two state cooperativity.

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