DECIPHERING THE FOLDING KINETICS OF TRANSMEMBRANE HELICAL PROTEINS

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Abstract

Nearly a quarter of genomic sequences and almost half of all receptors that are likely to be targets for drug design are integral membrane proteins. Understanding the detailed mechanisms of the folding of membrane proteins is a largely unsolved, key problem in structural biology. Here, we introduce a general model and use computer simulations to study the equilibrium properties and the folding kinetics of a $C_\alpha$-based two helix bundle fragment (comprised of 66 amino-acids) of Bacteriorhodopsin. Various intermediates are identified and their free energy are calculated together with the free energy barrier between them. In 40% of folding trajectories, the folding rate is considerably increased by the presence of non-obligatory intermediates acting as traps. In all cases, a substantial portion of the helices is rapidly formed. This initial stage is followed by a long period of consolidation of the helices accompanied by their correct packing within the membrane. Our results provide the
framework for understanding the variety of folding pathways of helical trans-
membrane proteins.
Considerable effort has been expended to understand the dynamics of the folding and biological functionality of proteins. Whereas the behavior of small water soluble globular proteins is reasonably well understood both experimentally and theoretically\textsuperscript{2}, much less is known about membrane proteins (MP)\textsuperscript{4–7} that cross biological membranes. Transmembrane proteins (TMP) are the most important and best studied class of MP\textsuperscript{4–8}. They are characterized by the presence in their primary structure of long segments (20 – 30) of amino acids with a high degree of hydrophobicity. In the native structure, these correspond to the transmembrane segments which are inserted in the lipidic interior of the membrane\textsuperscript{9}. These segments are predominantly made up of α-helices and β-sheets. The stability of α-helices and β-sheets inside the membrane follow from the formation of hydrogen bonds between the backbone atoms – other possibilities are excluded within the apolar environment\textsuperscript{4,10}.

Phenomenological models have proved to be powerful for interpreting experimental data. The most common of these is the Two-Stage model based on experimental evidence that the folding of TMP occurs in two stages. In the first stage, α-helices and β-sheets are formed with the full native state structure being formed in a distinct second stage\textsuperscript{10}. A more refined model\textsuperscript{4} takes into account four main steps: partitioning, folding, insertion and association. Recently, Pappu et al.\textsuperscript{11} have used a potential smoothing algorithm to predict transmembrane helix packing in good accord with experimental data.

Milik and Skolnick\textsuperscript{12,13} have carried out careful Monte Carlo studies of the insertion of peptide chains into lipid membranes and have proposed a new hydropathy scale based on experimental data obtained by studying the interactions of tripeptides with phospholipid membranes\textsuperscript{14} and the self-solvation effect in protein systems\textsuperscript{15}. Recently Wimley and White\textsuperscript{16} have designed transmembrane peptides that spontaneously insert across bilayers but yet have measurable monomeric water stability, opening the way for the determination of the thermodynamic cost of partitioning hydrogen bonded peptide bonds into the membrane hydrocarbon core.

The Monte-Carlo results of Milik and Skolnick\textsuperscript{12,13} are in good accord with Engelman and Steitz’s helical hairpin hypothesis\textsuperscript{17} further extended by Jacobs and White\textsuperscript{5,18}. The unfolded
chain is first adsorbed onto the membrane interface, driven mostly by the hydrophobic
effect and electrostatic lipid-protein interactions. A polypeptide chain has a greater
possibility, while anchored to the interface, of saturating its internal hydrogen bonds and
forming helices. Such helical fragments have a greater propensity to subsequently diffuse
into the lipid phase.

A detailed study of TMP has not yet been possible because little is known about the inter-
actions between amino acids inside the membrane or between them and the lipid molecules.
Here, we adopt a simple, yet powerful, strategy for attacking the folding properties of TMP
that circumvents this shortcoming. Our novel approach is based on extensive studies of
the folding of globular proteins which have underscored the important role played by the
topology of the native state in controlling both the functionality and the main features of
the folding process. Nature uses a rich repertory of twenty kinds of amino acids with some-
times major and at other times subtle differences in their interactions with the solvent and
with each other in order to design sequences that fit the putative native state with minimal
frustration. Thus a fruitful and general strategy for the study of protein folding would be
to extract information on the folding process directly from the topology of the native state.

Our study here focuses on the folding process by using a tractable approach (described
in the Methods Section) that by-passes the details of the complex interactions of the protein
in the lipid enviroment by introducing effective potentials, induced by the presence of the
membrane and the associated interface region, that stabilize the native state structure. The
validity of the approach based on the native state topology, in the case of globular pro-
teins, has been confirmed a posteriori from the agreement between theory and experimental
findings. The approach proposed here is similar in spirit and ought to be a tool and a
guide for the difficult experimental situation of TMP. Our model allows a complete char-
acterization of the thermodynamics and the dynamics of the full folding process. Due to the
small number of degrees of freedom involved, the dynamics of the system can be simulated
for the full folding process. Moreover, the free energies of the most relevant intermediate
states and free energy profiles along the reaction paths connecting them can be explicitly
calculated by thermodynamic integration (see Methods). Thus the model is able to quantitatively discriminate between the possible reaction paths envisaged for the insertion process of TMP across the membrane, a feature that is not an obvious consequence of the structure of the model. Where there is overlap, our model captures the qualitative features of the earlier simulations of Milik and Skolnick.

The TMP we considered is made up of the first 66 amino acids of bacteriorhodopsin consisting of two $\alpha$-helices (Fig. 1a). It has been shown that the first two helices of bacteriorhodopsin can be considered as independent folding domains. Furthermore, the side-by-side interactions between transmembrane helices play a key role in the stabilization of the protein structure.

Our studies were carried out using a Monte Carlo algorithm that has been proven to be extremely efficient for interacting hetero-polymers (Methods). The behaviour of the structural similarity between the system equilibrated at temperature $T$ (measured in dimensionless units) and the native state is shown in Figure 1b in terms of the average fraction of native state contacts as a function of $T$ and partitioned depending on their positions with respect to the membrane. The three curves correspond respectively to the average fraction of native contacts inside ($q_m$), outside ($q_b$) and across ($q_s$) the membrane (see Methods). All these curves, well separated at high T, collapse for $T$ below the transition temperature $T_C \sim 0.6$, indicating a cooperative effect in the folding. On monitoring the free energy as a function of the energy around $T_C$, one observes additional local minima (besides those corresponding to the unfolded and folded states) suggesting the presence of an intermediate.

The intermediate is characterized by having the two helices almost completely formed but not yet correctly inserted across the membrane. A metastable state in which the protein exists at the membrane interface ought to be expected on general grounds. Indeed a generic heteropolymer with hydrophobic and hydrophilic amino acids, of which a TMP is a particular case, has a favorable conformation which is localized near surfaces between two selective media (the outside and the inside part of the membrane in the present case). At not too high temperatures, the gain in energy to place hydrophobic/hydrophilic protein segments in
their preferred environment compensates the entropy loss for being localized at the interface with respect to remain in the bulk phase. Thus, even though our model does not explicitly contain information on the character of the amino acids, it is able to predict this feature.

The presence of these extra minima suggests that non-constitutive membrane proteins would fold with multi-state kinetics corresponding to on-pathway intermediates. To establish their nature of and their influence on the dominant folding pathways, we have performed a detailed analysis of the folding kinetics. Each independent kinetic folding simulation was started with the equilibrated denaturated state at $T^* = 2.5$. The protein is placed initially outside the membrane in the interface region at a distance comparable to the average size of the denatured protein and then suddenly quenched to a temperature ($T = 0.4$) well below the transition temperature. This case simulates the folding kinetics of non-constitutive membrane proteins, i.e. proteins that do not need a translocon providing a ’tunnel’ through which the protein is injected into the lipid bilayer. Folding to the native state occurs mainly through the states depicted in Figure 2a with the dominant pathways shown in Figure 2b.

In all the pathways, the system goes from the unfolded state, $U$ to state $HI$ in which 80% of the secondary structure is formed (see $q$ in Figure 3c) and disposed horizontally along the interface. The free energy of this state (measured with respect to the free energy of the fully folded state) is $\sim 2.4 T_C$. This state corresponds to the formation of around 70% of the membrane contacts. The average time $\tau_{HI}$ to reach state $HI$ is of the order of 500 Monte Carlo steps (see Figures 3 and 4; each Monte-Carlo step corresponds to 50000 attempted local deformations.). State HI turns out to be an obligatory on-pathway intermediate of the folding kinetics for non-constitutive MP in agreement with the general argument mentioned above. Once the protein reaches state $HI$, it undergoes a relatively slow process of self-arrangement in order to insert and assemble the secondary structures across the membrane. This process is the rate-limiting step of the folding process, since it involves the translocation, through the lipidic layer, of a substantial number of hydrophilic residues. Among the possible pathways, starting from $HI$, the most frequent (60% of the cases) and the fastest turn out to be $U \rightarrow HI \rightarrow HV \rightarrow N$. A quantitative characterization of this dominant pathway
is presented in Figures 3 (for a single folding process) and 4 (as an average over 40 folding processes). The intermediate $HV$ is characterized by having one $\alpha$ helix inserted across the membrane and is reached in an average period corresponding to a significant fraction of the total folding time (see Figure 3). The free energy in this state is $\sim 0.98 \ T_C$. The free energy barrier between $HI$ and $HV$ is at $\sim 4.31 \ T_C$ (hence, the rate constant of the transition $HI \rightarrow HV$ is proportional to $k_{HI \rightarrow HV} = \exp\left(-\frac{(4.31 - 2.4) \ T_C}{T}\right)$). The full free energy profile versus a reaction coordinate is shown in Fig. 5. The last part of the folding process corresponds to the insertion of the second helix and the assembly of the two secondary structures into the native state structure. This process lasts approximately one third of the folding time along the pathway $U \rightarrow HI \rightarrow HV \rightarrow N$. The quasistatic free energy barrier between $HV$ and the folded state is $\sim 1.66 \ T_C$. The rate constant of the transition $HV \rightarrow N$ is, therefore, proportional to $\exp\left(-\frac{(1.66 - 0.98) \ T_C}{T}\right)$. These results are consistent with the time scales observed in the unconstrained folding dynamics. At the end, the protein is completely packed, ($q_m$ saturates to 1 (Figures 3a and 4a)) and the helices are correctly positioned across the membrane (note the second jump in the $z$ coordinate of the center of mass in Figures 3b and 4b).

Much slower dynamics can occur when non-obligatory intermediates are visited by the system. These long lived states ($\{I\}$ in Figure 2a) involve a distribution of misfolded regions that trap the system and are characterized by having most of the inter-helical contacts formed (assembly of the secondary structures) but with the two $\alpha$-helices still incorrectly positioned. Note, for example, that in states $\{I\}$, only transmembrane contacts and some contacts outside the membrane are misplaced and they account for only a small fraction of the native state energy. For this reason, in the states $\{I\}$, the free energy is $\sim 1.44 \ T_C$, only slightly higher than the free energy of $HV$. The folding can proceed from $\{I\}$ either by disentangling the two helices and passing through the obligatory intermediate $HV$, or by the simultaneous translocation through the membrane of the two helices. These processes, however, entail the crossing of a big free energy barrier ($\sim 5.18 \ T_C$ for the first process and 6.1 for the second) and happen with low probability. Indeed, at sufficiently
low temperatures, the loss in energy of the interhelical contacts is not compensated by the gain in the configurational entropy due to the uncoupling of the $\alpha$-helices. Thus below the folding temperature, I-states act as trapping regions for the system and when trapped, the protein spends most of the time during folding in this state.

In summary, we have presented detailed calculations of helical transmembrane proteins leading to a vivid picture of the folding process. Our strategy relies on the dominant role played by the topology of the native state structure and by the effective geometry imposed by the membrane and provides a picture which would be expected to be quite accurate for well-designed sequences that are a good fit to the target native state conformation. It is interesting to note that, with our choice of the parameters, the pathway in which the helices assemble outside the membrane and are inserted later is unlikely to occur.

Models based on the topology of the native state structure have been remarkably successful\textsuperscript{25–28} in correctly describing the main features of the folding process determined in experiments\textsuperscript{2,23,24,29–31} for various globular proteins. A similar approach has been generalized here to the almost virgin field of transmembrane proteins where experiments are rather difficult\textsuperscript{16,4,7}. Our findings do not depend on the precise values of the $\epsilon$ parameters introduced in the model underscoring the robustness of the results. Our approach predicts a folding process involving multiple pathways with a dominant folding channel. The simplicity of our model allows for a quantitative description of all the pathways since we can monitor the correct/uncorrect formation of native contacts and compute free energy profiles. Further details not captured by the present approach arising from amino-acid specific interactions among themselves, with the solvent and in particular with the interior of the membrane may of course change the quantitative nature of the results. However, our model, which captures the bare essentials of a membrane protein, ought to provide a zeroth order picture of the folding process. Also, as experimental data becomes available, the results could be benchmarked with models of this type to glean the other factors that matter.
I. METHODS

We represent the residues of the membrane protein as single beads centered in their $C_\alpha$ positions. Adjacent beads are tethered together into a polymer chain by a harmonic potential with the average $C_\alpha - C_\alpha$ distance along the chain equal to $3.8\text{Å}$. The membrane is described simply by a slab of width $w = z_{\text{max}} - z_{\text{min}} = 26\text{Å}$. Two non-bonded residues $(i, j)$ form a contact if their distance is less than $6.5\text{Å}$. In the study of globular proteins, the topology of the native state is encoded in the contact map giving the pairs $(i, j)$ of non-bonded residues that are in contact. Here, in addition, the locations of such pairs with respect to the membrane becomes crucial. The contacts are divided into three classes: membrane contacts where both $i$ and $j$ residues are inside the membrane, interface contacts with $i$ and $j$ in the interface region outside the membrane and surface contacts with one residue inside the membrane and the other outside. Thus a given protein conformation can have a native contact but improperly placed with respect to the membrane (misplaced native contact). The crucial interaction potential between non-bonded residues $(i, j)$ is taken to be a modified Lennard-Jones 12-10 potential:

$$\Gamma(i, j) = \begin{cases} 5 \left( \frac{d_{ij}}{r_{ij}} \right)^{12} - 6 \left( \frac{d_{ij}}{r_{ij}} \right)^{10} + 5 \Gamma_1(i, j) \left( \frac{d_{ij}}{r_{ij}} \right)^{12} & \text{if } (i, j) \text{ is not a contact in the native state,} \\ \epsilon_1 & \text{if } (i, j) \text{ is a contact in the native state but not at the proper location,} \\ \epsilon & \text{if } (i, j) \text{ is a native state contact in the proper region.} \end{cases}$$

(1)

The matrices $\Gamma(i, j)$ and $\Gamma_1(i, j)$ encode the topology of the TMP in the following way: if $(i, j)$ is not a contact in the native state $\Gamma(i, j) = 0, \Gamma_1(i, j) = 1$; if $(i, j)$ is a contact in the native state but not at the proper location (i.e. a misplaced contact) $\Gamma(i, j) = \epsilon_1, \Gamma_1(i, j) = 0$; if $(i, j)$ is a native state contact in the proper region $\Gamma(i, j) = \epsilon, \Gamma_1(i, j) = 0$. This model is intended to describe the folding process in the interface and in the membrane region. Our interaction potential (similar in spirit to a well known model for globular proteins (see also other approaches that model helix formation)) assigns two values to the energy associated with the formation of a native contact, $\epsilon$ and $\epsilon_1$.

The model captures the tendency to form native contacts. In addition, in order to account for the effective interactions between the membrane and the protein, the model
assigns a lower energy, \(-\epsilon\), to the contact which occurs in the same region as in the native state structure compared to \(-\epsilon_1\) when the contact is formed but in the wrong region of space. This feature proves to be crucial in determining the mechanism of insertion of the protein across the membrane in order to place all native contacts in the same regions as in the native state. Even though the interaction potential is simple and intuitively appealing, it is not possible to simply guess (without detailed calculations) the folding mechanism and quantitatively determine the probability of occurrence of the various folding pathways.

When \(\epsilon = \epsilon_1\), the protein does not recognize the presence of the interface-membrane region and the full rotational symmetry is restored (the system behaves like a globular protein). The difference in the parameters \((\epsilon - \epsilon_1)\) controls the amount of tertiary structure formation outside the membrane. When the difference is small, the protein assembles almost completely outside the membrane and the insertion process would be diffusion limited. Our results are independent of the precise values of the energy parameters \(\epsilon\) and \(\epsilon_1\) \((\epsilon > \epsilon_1)\) as long as they are not too close to each other.

We report here the results of simulations with \(\epsilon_1 = 0.1\) and \(\epsilon = 1\). \(r_{ij}\) and \(d_{ij}\) are the distance between the two residues \((i, j)\) and their distance in the native configuration, respectively. In order to account for the chirality of the TMP, a potential for the pseudodi-hedral angle \(\alpha_i\) between the \(C_\alpha\) atoms in a helix corresponding to four successive locations is added which biases the helices to be in their native state structure.

The thermodynamics and the kinetics of the model were studied by a Monte Carlo method for polymer chains allowing for local deformations. The efficiency of the program (usually low for continuum calculations) has been increased by full use of the link cell technique and by the multiple Markov chain method, a new sampling scheme, which has been proven to be particularly efficient in exploring the low temperature phase diagram for polymers. In our simulation 20 different temperatures ranging from \(T = 2\) to \(T = 0.17\) have been studied. The free energy is calculated by reweighting the different temperatures with the Ferrenberg-Swendsen algorithm.

The free energy difference \(\mathcal{F}_B - \mathcal{F}_A\) between two states A and B has been estimated as
the reversible work that has to be done in order to go from $A$ to $B$. Hence, denoting by $x(\lambda)$ a reaction coordinate connecting $A$ and $B$ (for $\lambda = 0$ and $\lambda = 1$ the system is in $A$ and $B$ respectively), and by $\langle \cdot \rangle_\lambda = \langle \delta(x - x(\lambda)) \cdot \rangle$, the canonical average at fixed reaction coordinate,

$$
\mathcal{F}_B - \mathcal{F}_A = \int_0^1 d\lambda \langle F \rangle_\lambda \cdot \frac{dx(\lambda)}{d\lambda} \simeq \sum_i \langle F \rangle_{\lambda_i} \cdot \left. \frac{dx(\lambda)}{d\lambda} \right|_{\lambda = \frac{\lambda_i + \lambda_{i+1}}{2}} (\lambda_{i+1} - \lambda_i)
$$

(2)

where $F$ is the force and $\{\lambda_i, i = 1, \ldots\}$ is a suitably dense partition of the interval $(0, 1)$. The average value $\langle F \rangle_{\lambda_i}$ at each $\lambda_i$ is computed by a long (more than 5000 steps) Monte Carlo run performed with dynamics satisfying the constraint $x = x(\lambda_i)$. The free energy differences obtained with this method are accurate to within $\sim 0.1 T_C$ for the various states whereas the free energy barriers are accurate within $\sim 0.5 T_C$. This error takes into account possible hysteresis effects due to the finite simulation time.
REFERENCES

1 Nature Structural Biology 6, 1-2 (1999).

2 Fersht, A. R., W. H. Freeman, New York (1999).

3 Karplus, M. & Sali, A. Curr. Opin. Struct. Biol. 5, 58-73 (1995).

4 White, S. H. & Wimley, W. C. Ann. Rev. Biophys. Biomol. Struct. 28, 319-365 (1999).

5 Ostermeier, C. & Michel, H. Curr. Opin. Struct. Biol. 7, 697-701, (1997).

6 von Heijne, G. Prog. Biophys. Molec. Biol. 66, 2, 113-139 (1996).

7 Booth, P.J. Folding & Design, R85-R92 (1997).

8 Biggin, P. C. & Sansom, M. S. P. Biophysical Chemistry 76, 161-183 (1999).

9 Deber, C. M. & Goto, N. K. Nature Structural Biology 3, 815-818 (1996).

10 Popot, J. L. & Engelman, D. M. Biochemistry 29, 4031-4036 (1990).

11 Pappu, R. V., Marshall, G. R. & Ponder, J. W. Nature Structural Biology 6, 50-55 (1999).

12 Milik, M. and Skolnick, J. Proc. Natl. Acad. Sci. USA 89, 9391-9395, (1992)

13 Milik, M. and J. Skolnick. Proteins: Funct. Stru. Gen. 15,10-25, (1993)

14 Jacobs,R. E. & White, S.H. Biochemistry 26, 6127-6134 (1987)

15 Roseman, M. A., J. Mol. Biol. 200, 513-522 (1988).

16 Wimley, S.C. & White, S.H Designing Transmembrane α-helices That Insert spontaneously preprint. University of California, Irvine (2000)

17 Engelman, D.M. & Steitz, T.A. Cell, 23, 411-422 (1981).

18 Jacobs, R.E. & White, S.H. Biochemistry, 28, 3421-3427 (1989).

19 Jacobs, R.E. & White, S.H. Biochemistry, 25, 2605-2612 (1986).
20 Pinheiro, T. J. T., Elöve, G.A., Watts, A. and Roder, H. *Biochemistry* **36**, 13122-13132 (1997)

21 Rankin, S. E., Watts, A. and Pinheiro, T.J.T. *Biochemistry* **37**, 12588-12595 (1998)

22 Bryson, E. A., Rankin, S.E., Carey, M. Watts, A. and Pinheiro, T.J.T. *Biochemistry* **38**, 9758-9767 (1999)

23 Fersht, A. R. *Current Opinion in Struct. Biology* **5**, 79-84 (1995)

24 Fersht, A. R. *Current Opinion in Struct. Biology* **7**, 3-9 (1997)

25 Micheletti, C., Banavar, J. R., Maritan, A. & Seno, F. *Phys. Rev. Lett.* **82**, 3372-3375 (1999).

26 Munoz, V. & Eaton, W. A. *Proc. Natl. Acad. Sci.* **96**, 11311-11316 (1999).

27 Galzitskaya, O. V. & Finkelstein, A. V. *Proc. Natl. Acad. Sci.* **96**, 11299-11304 (1999).

28 Alm, E. & Baker, D. *Proc. Natl. Acad. Sci.* **96**, 11305-11310 (1999).

29 Chiti, F., Taddei, N., White, P. M., Bucciantini, M., Magherini, F., Stefani, M. and Dobson, C. M. *Nature Structural Biology* **6**, 1005, (1999).

30 Martinez, J. C. and Serrano, L. *Nature Structural Biology* **6**, 1010, (1999).

31 Riddle, D. S., Grantcharova, V. P., Santiago, J. V., Alm, E., Ruczinski, I. and Baker, D. *Nature Structural Biology* **6**, 1016, (1999).

32 Bryngelson, J. D. and Wolynes, P. G. *Proc. Nat. Acad. Sci. USA* **84**, 7524, (1987).

33 Kahn, T. W., Sturtevant, J.M. and Engelman, D.M. *Biochemistry* **31**, 8829-8839 (1992)

34 Kahn, T. W. & Engelman, D.M. *Biochemistry* **31**, 6144-6151 (1992)

35 Garel, T., Huse, D.A., Leibler, L. & Orland, H. *Europhys. Lett.* **8**, 9-12 (1989)

36 Maritan, A., Riva, M.P. & Trovato, A. *J. Phys. A: Math. Gen.* **32**, L275-L280 (1999)
37 Taketomi, H., Ueda, Y. & Go, N. *Int. J. Pept. Protein Res.* **7**, 445-459 (1975).

38 Guo, Z. & Thirumalai, D. *Journal of Molecular Biology* **263**, 323-343 (1996)

39 Takada, S., Luthey-Schulten, Z. & Wolynes, P.G. *Jour. of Chem. Phys.* **110**, 11616-11629 (1999)

40 Geroff, I., Milchev, A., Binder, K. & Paul, W. *J. Chem. Phys.* **98**, 6256-6539 (1993).

41 M. C. Tesi, van Rensburg, E.J., Orlandini, E. & Whittington, S. G. *J. Stat. Phys.* **29**, 2451-2463 (1996).

42 Ferrenberg, A. M. & Swendsen, R. H. *Phys. Rev. Lett.* **63**, 1195-1198 (1989).

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II. FIGURE LEGENDS

Figure 1: Structure and thermodynamics of the helical transmembrane protein.

a) Ribbon representation of the two-helix fragment of bacteriorhodopsin formed by the first 66 amino-acids. The part inside the membrane (determined by using the neural network learning algorithm available at http://www.embl-heidelberg.de/Services/sander/predictprotein/) is shown in red, the part above (below) the membrane in blue (green).

b) Average equilibrium fraction of native contacts outside, $q_b$, inside, $q_m$, and across, $q_s$, the membrane as a function of the temperature $T$. All these quantities are expressed in energy unit of $\epsilon$ (see Methods). The folding transition temperature $T_C$ when all the curves cross the value $1/2$ is around 0.6. This value is in accord with the temperature of the heat capacity maxima.

Figure 2: Schematic representation of states encountered by non-constitutive proteins during the folding process.

In a) the red cylinders denote $\alpha$-helices that reside within the membrane in the native state. The region inside the membrane is in turquoise whereas the rest represents the interface region in which the folding process starts. State $U$ denotes the denatured state of the protein, $HO$ is a state in which the helices have been formed but are not yet inside the membrane whereas $HI$ corresponds to a similar state but with the helices completely embedded in the membrane without any inter-helical contacts. Usually the helices form and enter into the membrane separately. $HV$ denotes an obligatory intermediate and $N$ depicts the native state. The state $\{I\}$ represents an ensemble of long lived conformations in which helices are formed inside the membrane with several inter-helical contacts, but with the two $\alpha$-helices still incorrectly positioned. This conformations differ in term of packing efficiency of the helices. The state $\{I\}$ is not obligatory for the folding kinetics. In b) the schematic pathways to the native state are shown. In the most directed path, the entropy decreases on going from $U$ to $N$. From $HI$ to $HV$ the entropy loss of one helix is not compensated by a corresponding energy gain until both helices become vertical. This is the principal origin.
of the high free energy barrier between the state $HI$ and the native state.

**Figure 3:** Typical time dependence of different parameters as a function of the Monte-Carlo steps for the pathway $U \rightarrow HI \rightarrow HV \rightarrow N$. Fraction of native contacts inside the membrane (a), normalized $z$-coordinate of the center of mass of the protein (with respect to that of the native state conformation) (b) and overall fraction of native helical contacts (c). Each Monte-Carlo step corresponds to 50000 attempted local deformations. The transition from state $HI$ to state $HV$ is signalled by a sharp jump of the position of the center of mass. Note that there is no perceptible sign of this transition in terms of newly formed native contacts. Most of the helical contacts are formed in the early stages of the folding. This fraction does not significantly increase until helices correctly assemble and the inter-helical contacts are formed. The $HV \rightarrow N$ transition is reached by a progressive zipperling of the horizontal and vertical helices. This zipperling is usually very quick (few MC steps) and is only slightly slowed down (see the plateau corresponding to $q_m \sim 0.9$ in a) when the trajectory passes through somewhat deformed conformations. (d) Protein conformations at different times during the folding. The colours red, green and blue have the same significance as in Figure 1a with the grey bonds being ones crossing the membrane.

**Figure 4:** Distribution of the fraction of native contacts inside the membrane (a) and of the normalized $z$-coordinate of the center of mass ($R_z = \frac{z_{cm}}{z_{cm,nat}}$) (b). The data were obtained using 40 independent kinetic simulations with pathway $U \rightarrow HI \rightarrow HV \rightarrow N$. The grey scale distribution indicates the probabilities at given times: darker points denote higher probability.

**Figure 5:** Free energy profiles along three reaction coordinates at $T=0.85 T_C$. The continuous lines are spline fits to the free energy data (crosses). To obtain free energy differences between two states we estimate the reversible work that has be done to go from one state to the other. For this purpose, we fix the $z$ coordinate of a specific residue in order to compute the canonical average of the force and then apply eq. (2) (See Methods). The free energy of the native state is defined to be equal to 0. (a) Free energy as a function of the $z$-coordinate of the 58-th residue ($z = 0$ corresponds to the middle of the membrane)
starting from HV; this forces the second helix to cross the membrane as the protein goes from HV to N; the local minimum at $z \approx 20$ corresponds to a state topologically equivalent to HV, with the helix containing the 58-th residue fully formed on the membrane interface but without any contact with the first helix (in HV some of the inter-helices contacts are already formed); (b) the 5-th residue is translocated across the membrane with the protein starting from state HI and proceeding to HV; (c) the same as in (b), but the initial state is I (see Fig. 2-a)
FIGURES

a) 

b) 

FIG. 1. Seno
FIG. 2. Seno
FIG. 3. Seno
FIG. 4. Seno
FIG. 5. Seno