Published in final edited form as:

J Phys Chem B. 2013 November 7; 117(44): 13748–13754. doi:10.1021/jp406925y.

On the Nature of the Apparent Free Energy of Inserting Amino Acids into Membrane through the Translocon

Anna Rychkova¹ and Arie Warshel^{2,*}

¹Stanford University, Department of Genetics, 365 Lasuen St, Littlefield Center MC2069, Stanford, CA 94305

²University of Southern California, Department of Chemistry, 3620 McClintock Ave, SGM 418, Los Angeles, CA 90089-1062

Abstract

The nature of the biological free energy scale (ΔG_{app}), obtained from translocon mediated insertion studies has been a major puzzle and the subject of major controversies. Part of the problem has been the complexity of the insertion process that discouraged workers from considering the feasible kinetic schemes and left the possible impression that ΔG_{app} presents some simple partition. Here we extend and clarify our recent analysis of the insertion problem using well-defined kinetic schemes and a free energy profile. We point out that although the rate constants of some steps are far from being obvious, it is essential to consider explicitly such schemes in order to advance in analyzing the meaning of ΔG_{app} . It is then shown that under some equilibrium conditions the kinetic scheme leads to a simple formula that allows one to relate ΔG_{app} to the actual free energy of partitioning between the water, the membrane and the translocon. Other options are also considered (including limits with irreversible transitions that can be described by linear free energy relationships (LFERs)). It is concluded that it is unlikely that a kinetic plus thermodynamic based analysis can lead to a result that identifies ΔG_{app} with the partition between the membrane and the translocon. Thus we argue that unless such analysis is presented it is unjustified to assume that ΔG_{app} corresponds to the membrane translocon equilibrium or to some other arbitrary definition. Furthermore, we point out that the presumption that it is sufficient to just calculate the PMF for going from the TR to the membrane and then to assume irreversible diffusive motion to water and for further entrance to the membrane, is not a valid analysis. Overall, we point out that it is important to try to relate ΔG_{app} to a well-defined kinetic scheme (regardless of the complication of the system) in order to determine whether the energies of inserting positively charged residues to the membrane is related to the corresponding ΔG_{app} . It is also suggested that deviations from our simple formula for equilibrium conditions can help in identifying and analyzing kinetic barriers.

Keywords

hydrophobicity scale; kinetic scheme; coarse-grained model

I. Introduction

The pioneering experimental studies of von Heijne group¹ explored the energetics of inserting amino acids into a membrane by constructing a model system of the double-spanning natural protein (bacterial leader peptidase) with engineered third hydrophobic

*Corresponding Author. Tel.: +1(213)740-4114; Fax: +1(213)740-2701; warshel@usc.edu.

segment (H-segment). The degree of membrane integration of the H segment was then determined by flanking the segment with two acceptor sites for N-linked glycosylation and evaluating the number of glycosylated sites and the apparent equilibrium constant, $K_{\text{app}} = f_{1g}/f_{2g}$ (where f_{1g} and f_{2g} are the fractions of singly and doubly glycosylated proteins, respectively). This value was then converted to the relevant apparent free energy, $\Delta G_{\text{app}} = -RT \ln K_{\text{app}}$, which was then decomposed to the contribution ΔG_{app}^i from each of the 20 naturally occurring amino acids placed in the middle of the H helix.

The exact meaning of ΔG_{app} remains unclear and even Hessa et al.¹ mentioned that the process of translocon-assisted insertion of peptides into the membrane is very complicated and probably does not represent a true thermodynamic equilibrium. They also warned that they do not try to imply such equilibrium. However, it has been assumed by many that ΔG_{app}^i is related to the energy of inserting the corresponding amino acid to the membrane. The implication of this assumption has continued to be a subject of significant controversies in particular because of the small estimate of the free energy of inserting charged residues into membranes. Various arguments were put forward to rationalize the discrepancies between the estimates of the insertion energy from ΔG_{app}^i and the values obtained from the free energy calculations²⁻⁶. These included the argument that the apparent energy corresponds to equilibrium between the translocon (TR) and the membrane rather than between water and the membrane^{5,6} as well as related proposals⁷ and in particular ref⁸ that unfortunately only calculate explicitly the TR membrane PMF. Unfortunately, none of the early considerations involved a detailed assumed kinetic scheme and a well-defined proposal for the assumed equilibrium or nonequilibrium relationship or the relevant set of barriers (although a tentative suggestion has been put forward in the SI of ref⁴).

Our recent work⁹ allowed us to estimate the free energy profile for the process of TR-assisted peptide insertion into membrane. The details of the emerging profiles turned out to be quite complicated and are still subjected to further refinement. Nevertheless, the consideration that emerged during this work (and in particular the actual attempt to generate a well-defined profile) has guided us in obtaining interesting conclusions, where some of them seem to be robust and not so sensitive to the details of the free energy barriers. However, our previous work was mainly dedicated to estimating the key free energy barriers; it has not provided a sufficiently clear discussion of the nature of ΔG_{app} (which was not the main subject of our work). Thus we find it instructive to clarify and extend our considerations below. This will be done by both presenting the overall complexity of the problem and extending the discussion of an instructive limiting case that leads to a simple quasiequilibrium result. We also clarify that oversimplified assumption that ΔG_{app} is equal to the membrane equilibrium is not justified.

II. Analyzing the meaning of ΔG_{app} using well defined kinetic scheme

In order to explore the meaning of ΔG_{app} it is essential to consider a well-defined kinetic scheme with all the possible paths. Obviously we may end up with unknown rates for the assumed elementary steps, but without such a framework it is very hard to progress in a logical way. Thus we start our analysis with the schematic kinetic diagram presented in Fig. 1, where we consider the main feasible paths involved in the insertion experiment. In doing so we add to our previous description⁹ an additional step that divide the system in water into two main subsystems, separating the early insertion of the H-segment into water (W) from a subsequent step where all the rest of the protein is transferred to water and finally get glycosylated (W_g)¹⁰. We also present in Fig. 2 a schematic energy diagram for the paths described in Fig. 1. Furthermore, we consider in Fig. 3 a significantly more complex diagram with more detailed pathways (this diagram and its implications will be discussed in section III).

Although the mechanistic options can be somewhat overwhelming (e.g. Fig. 3), it is instructive to consider first the simplest option, obtained by simplifying Fig. 3 drastically, and assuming that the forward and backward rate constants for the transfer from the TR to both membrane (M) and water (W) are sufficiently large (small barriers), so that we have equilibration for each process (this assumption is likely to be true (see ref⁹ but cannot be taken as an absolute fact). We also consider a simplifying situation where the activation barriers for moving from W to W_g and from M to M_g are similar. We note that both states W_g and M_g are combinations of several states (as is clear from Fig. 3). Under the above assumption we will obtain a partition between the H-segment in water and the H-segment in both the TR and the membrane. Using TR, M and W for both the state names and their corresponding concentrations we can write:

$$K_1 = W/TR \quad K_2 = M/TR \quad TR + M + W = 1 \quad (1)$$

We can also write

$$K_{app} = (M + TR)/W \quad (2)$$

Note that here we added the fraction that is still in the TR as part of the membrane since we cannot separate them experimentally. Solving Eqs. 1 and 2 we get

$$K_{app} = (1 + K_2)/K_1 \quad (3)$$

This important result (which has not been pointed out before the work of ref⁹) corresponds to the water/membrane equilibrium and not to the membrane/TR equilibrium. In the limit where K_2 and K_1 are significantly larger than 1 (only small fraction is in the TR), we find that K_{app} can be approximated by $K_2/K_1 = M/W$. In the other limit of $K_2 \ll 1$ we find that $K_{app} = 1/K_1 = TR/W$. This limit ($K_2 \ll 1$) can occur in principle when we have an ionized residue in the membrane. This does not mean that the H-segment is stacked in the TR since it can move to water. Interestingly, when K_{app} is approximately 1, which is the range chosen in designing the H-segment for studying ΔG_{app} , we can have the above $K_2 \approx K_1 \gg 1$ or $K_2 \ll 1$ and $K_1 \approx 1$ limits considered above and intermediate cases that will be also discussed here. None of this limits leads to $K_{app} = K_2 = M/TR$ and thus it seems to us that the recent attempts⁵⁻⁷ to invoke the membrane/TR equilibrium as the factor that determines ΔG_{app} are not justified. This problem is compounded by the fact that none of the suggestions to identify ΔG_{app} with the M/TR equilibrium have been supported by a kinetic or thermodynamic analysis.

Now let ask ourselves when the experimentally determined apparent free energy, ΔG_{app} , actually represents the free energy of moving the residue from water to membrane, $\Delta G_{W \rightarrow M}$. This question can be formulated using:

$$\Delta G_{W \rightarrow M} = -RT \ln(K_2/K_1) = -RT \ln(M/W) \quad (4)$$

and

$$\Delta G_{app} = -RT \ln K_{app} = -RT \ln((1 + K_2)/K_1) \quad (5)$$

where we find out that ΔG_{app} is equal to $\Delta G_{W \rightarrow M}$ when $K_2 \gg 1$, or $M \gg TR$. This condition can be fulfilled for residues with $\Delta G_M < \Delta G_{TR}$, which is likely to be true for hydrophobic residues that prefer to stay in the membrane. This indicates that the hydrophobicity scale may work well for the hydrophobic residues. In fact, some theoretical studies support this finding¹¹. The analysis is complicated by the possibility that the H-segment in water (W_g)

might be stabilized by the interaction with the membrane, with the C terminal domain of the protein, or with other proteins.

Another interesting limit that will also be discussed here in a tentative way is the limit where both $\Delta G_{TR \rightarrow M}$ and $\Delta G_{TR \rightarrow W}$ are very negative so the splitting to M and W is irreversible. In this case we can use our linear free energy relationship (LFER) idea¹² of correlation between the change in equilibrium constant and the change in the rate constant. Here with the expected LFER (see appendix of ref⁹) ($\Delta \ln K = \alpha \Delta \ln k$) we can start with

$$\Delta K_{app} \approx \Delta(k_{TR \rightarrow M}/k_{TR \rightarrow W}) = \Delta \exp\{-(\Delta g_{TR \rightarrow M}^\ddagger - \Delta g_{TR \rightarrow W}^\ddagger)\beta\} \quad (6)$$

where Δ is the change due to changes in the H helix, and use the LFER to obtain

$$\alpha \Delta \ln(k_{i \rightarrow j}) = -\alpha(\Delta \Delta g_{i \rightarrow j}^\ddagger \beta) = \Delta \ln K_{i \rightarrow j} = -\Delta \Delta G_{i \rightarrow j} \beta \quad (7)$$

so that with Eqs. 6 and 7 we obtain

$$\Delta \ln K_{app} \approx -\Delta(\Delta g_{TR \rightarrow M}^\ddagger - \Delta g_{TR \rightarrow W}^\ddagger)\beta = -\Delta(\Delta G_{TR \rightarrow M} - \Delta G_{TR \rightarrow W})\beta/\alpha \quad (8)$$

The present work uses a CG model that describes the main chains by an explicit model and represents the side chains by a simplified united atom model. The model includes a unique treatment of the electrostatic energy including the self-energy that gives its reliable features. The model has been continuously refined^{13,14} and the most recent modifications and parameters are given in the MOLARIS manual¹⁵. The CG treatment used to obtain the energy values in Table 1 has been described in ref⁹. This CG model allows us to estimate the free energy of the TR-assisted H-segment membrane insertion and translocation, and more importantly (for the purpose of this paper) to demonstrate what is needed for the analysis of ΔG_{app} . That is, using our CG model we evaluated the energetic of the H-segments with different central residues in the TR, M and W states (see results in Table 1). Using the above consideration for the hydrophobic residues we can estimate the apparent free energy for replacing the central Leu by Ile. Here in the limit of $M \gg TR$ $\Delta G_{app} = \Delta G_{W \rightarrow M}$ so that

$$\Delta \Delta G_{app}(Leu \rightarrow Ile) = \Delta \Delta G_{W \rightarrow M}(H_{Ile}) - \Delta \Delta G_{W \rightarrow M}(H_{Leu}) = 0.73 \text{ kcal/mol} \quad (9)$$

where H_{Ile} and H_{Leu} indicate the H-segment with central Ile and Leu, respectively. This value is close to the experimentally observed $\Delta \Delta G_{app}^{obs}(Leu \rightarrow Ile) = -0.05 \text{ kcal/mol}$. However our results cannot unambiguously distinguish between the limit of $M \gg TR$ and the limit $TR \gg M$ where $\Delta G_{app} = \Delta G_{W \rightarrow TR}$, since in this second limit we obtain a similar result:

$$\Delta \Delta G_{app}(Leu \rightarrow Ile) = \Delta \Delta G_{W \rightarrow TR}(H_{Ile}) - \Delta \Delta G_{W \rightarrow TR}(H_{Leu}) = 0.64 \text{ kcal/mol} \quad (10)$$

Most likely the results in the two limits are close to each other due to the small absolute value of the apparent free energy for hydrophobic residues.

The situation becomes more complex in the case of polar and ionized residues where $K_2/K_1 < 1$. For example, in the range of $K_2 \ll 1$ we have $K_{app} = 1/K_1 = TR/W$ and $\Delta G_{app} = \Delta G_{W \rightarrow TR}$. Now we have:

$$\Delta \Delta G_{app}(Leu \rightarrow Arg) = \Delta \Delta G_{W \rightarrow TR}(H_{Arg}) - \Delta \Delta G_{W \rightarrow TR}(H_{Leu}) \quad (11)$$

and using data from Table 1 we obtain $\Delta \Delta G_{app}(Leu \rightarrow Arg) = 20.05 \text{ kcal/mol}$. In this case we have equilibrium between water and the TR. This corresponds to the situation when Arg

is in the center of the membrane and is not stabilized by another helix. The conflict between the reasonable estimate of 20 kcal/mol for the energy of moving a charged Arg from water to non-polar environment and low experimental ΔG_{app} for Arg has been the origin of the controversy in the field. However, as we show here the partition measurements cannot determine cases of extreme instability in the membrane since the H-segment will be in the TR and the experimental separation probably cannot distinguish between the cases with the glycosylated H-segment in the membrane or in the TR.

On the other hand, in the range when Arg is more stable in the center of the membrane than in the TR due to interaction with other helices^{3,4} or because of a combination of snorkeling, helix tilting and helix sliding effects^{2,16} we can have $K_2 \approx K_1 \gg 1$ and thus $\Delta G_{app} = \Delta G_{W \rightarrow M}$. Thus we get:

$$\Delta \Delta G_{app}(Leu \rightarrow Arg) = \Delta \Delta G_{W \rightarrow M}(H_{Arg}) - \Delta \Delta G_{W \rightarrow M}(H_{Leu}) \quad (12)$$

and using the data from Table 1 we obtain $\Delta \Delta G_{app}(Leu \rightarrow Arg) = 17.22 \text{ kcal/mol}$.

Interestingly, our results indicate that Arg is unstable in both the TR and membrane. This indicates that additional effects (e.g. interaction with other proteins, side chain tilting, water penetration and others) are probably essential in facilitating the insertion of charged residues into the membrane. Of course, in the case of groups with pKa which is not so different than 7 we will also have neutralization (e.g. the Glu going to GluH in our paper⁴).

III. Complications and Alternative Analyses

The above analysis has been based on the assumption of Fig. 2 that the barriers for going back and forth to W and M are not too high and on other simplifications. Of course, these assumption may be unjustified as clarified in our previous work⁹, thus the valid scientific direction is to explore the implications of different feasible limits. Thus, at the expense of significant complexity, we move to the more complete analysis of Fig. 3. The figure includes more realistic and complicated set of paths and detailed rough estimates of the energies of the different states and the corresponding barriers (based on our previous works^{9,17}). The figure can lead to the results of Eq. 5 if K_1 is similar to K'_1 and K_2 is similar to K'_2 . Overall, if the quasiequilibrium is established before and after the detachment of the ribosome we can still recover the trend of Eq.5. Interestingly, if the transfer to W_4 occurs much before the glycosylation and the energy of W_4 is lower than that of W_1 we may move to W_g without the option of returning to W_1 . This will force more molecules to move from M_1 to W_1 and change the quasiequilibrium. In principle the entire system can end up in W_g if the process would be allowed to go to completion.

Of course, other options are possible, and some limiting cases have been considered in ref⁹, but our main point is the need for a kinetic-based analysis and the fact that such an analysis leads to some important conclusions regardless of the current uncertainties.

IV. Dependence on the Translocon/H-segment Interaction

Some readers who do not accept our mathematical considerations as a valid way of exploring ΔG_{app} might assume that we proposed that this quantity should be identified with the water/membrane (M/W) partition. This, of course, ignores the clear message from Eq.1. In fact, a referee of this work argued that we cannot explain the observed dependence of ΔG_{app} on the TR nature that emerged for example from the experiments of ref¹⁸. Of course, this reflects the common assumption that what we think on the effect of mutations can be used as a scientific judgment instead of actually simulating the given effect. Since at present our studies are probably the only studies that provided a clear logical framework of asking

what is the meaning of ΔG_{app} , we find it instructive to consider here a very recent experiment that has been reported by Spiess and coworkers (Spiess' private communication). This study found that taking an H segment with 19 Ala and placing a cluster of 3 Leu at different points give different degrees of membrane integration. For example, moving from position 3 to position 14 lead to a change of the membrane integration from 90% to 40%.

These numbers can be converted to $K_{app}^{(3)}=9.0$ and $K_{app}^{(14)}=0.67$. A preliminary exploration of this experiment is reported in Fig 4. As seen from the figure the free energy inside the TR increases by about 2 kcal/mol.

In order to relate this finding to the new experiments we note that $K_{app}^{(3)} / K_{app}^{(14)} = 13.4$ thus $\Delta\Delta G_{14\rightarrow 3} = -RT\ln(K_{app}^{(3)} / K_{app}^{(14)}) = -1.56\text{kcal/mol}$.

Now, from Eq. 1 we obtain

$$K_{app} = (1 + K_2) / K_1 = M/W + TR/W = C + TR/W \quad (13)$$

Using the results of Fig. 4 we obtain in the limit of $C \ll 1$

$$\Delta\Delta G_{14\rightarrow 3} = -RT\ln((TR^{(3)} / TR^{(14)}) = \Delta\Delta G(TR_{14\rightarrow 3}) = -1.69 \quad (14)$$

In the limit $C \approx 1$ we obtain

$$\Delta\Delta G_{14\rightarrow 3} = -RT\ln[(1 + TR^{(3)} / W^{(3)}) / (1 + TR^{(14)} / W^{(14)})] \quad (15)$$

With $TR^{(3)} / W^{(3)} \approx 1$ and $TR^{(3)} / W^{(3)} \Delta 1$, we obtain $\Delta\Delta G_{14\rightarrow 3} \approx -RT\ln 2 = -0.4\text{kcal/mol}$. Finally, when $C \gg 1$ we obtain

$$\Delta\Delta G_{14\rightarrow 3} = -RT\ln[(M^{(3)} / W^{(3)}) / (M^{(14)} / W^{(14)})] = 0 \quad (16)$$

The limit of $C \ll 1$ seems unrealistic since we deal with a hydrophobic helix. However, as clarified in ref⁹ we actually do not know what is the free energy difference between state W and state M since the H-segment is on the membrane water boundary. Thus our point is that we clearly have cases when Eq. 1 reflects the energetics in the TR and that even Eq. 2 is not restricted at all to the case where ΔG_{app} corresponds to the W/M equilibrium. Clearly as shown in section III the kinetic diagram can be more complex and reproducing the experimental trend should require further progress in simulating the different barriers.

V. Concluding discussion

The elucidation of the origin of ΔG_{app} is important both for a better understanding of the TR-assisted insertion process and, hopefully, for gaining insight about the energetics of inserting amino acids into membranes. Here we expand and clarify our previous analysis emphasizing that it is crucial to attempt to relate ΔG_{app} to a well-defined kinetic and energy considerations. While it is impossible to reach at present a conclusive picture, our systematic analysis of ΔG_{app} provides an improved description of the actual nature of the corresponding biological scale. It is concluded that the interpretation of ΔG_{app} can be accomplished (in some limits) if we know the relative energy of moving from the TR to the membrane (this should not be confused with the idea that ΔG_{app} is determined by the TR to membrane equilibrium). For the case with $K_2 \gg 1$ we can use Eq. 4 to determine the W to M insertion energy.

Another important point is the fact that the present analysis is done in the limit where the barriers of moving from the membrane to the TR and from water (in state W) to the TR is

relatively low so we have an equilibration between states M, TR and W. This assumption (that should not be confused with the barrier for the A to TR transition in the initial penetration step) is reasonable but not certain. The case where the barriers are high has been discussed in ref⁹ and the corresponding kinetics can be easily incorporated into the analysis of ΔG_{app} . Furthermore, even the more complex situation considered in section III can be related to ΔG_{app} if the relevant barriers are known.

Another point that can lead to some misunderstandings is the possible perception that we postulate that the H segment can be trapped in the TR. However, our TR state includes any substate where the H segment is in or touching the TR including in the membrane/TR interface. The existence of such states cannot be excluded by the current glycosylation and is clearly consistent with Fig. 3. Similarly our M state includes the states where the H segment is attached to the other two helices as discussed in ref⁴. Even attempts to exclude this possibility⁵ are not at all conclusive (see discussion in ref⁹).

It should be noted that our simplest mechanistic option does not reproduce exactly the observed ΔG_{app} in particular considering the remarkable stability in the corresponding observed trend. Thus we have not provided a unique solution to the meaning of ΔG_{app} but rather outlined the way this scale can be analyzed once all the kinetic steps are understood. Perhaps more encouraging is the fact that our analysis indicated clearly that some assumptions about the origin of ΔG_{app} cannot be supported by logical kinetic analysis.

In view of input received from a referee of this work we like to clarify that we appreciate the insight of previous works that questioned the idea that ΔG_{app} corresponds to the TR/W equilibrium. However, the suggestions that ΔG_{app} may involve complex effects does not qualify it as an analysis of the origin of this key observable. Here our insistence on defining ΔG_{app} by kinetic diagrams is a reflection of the belief that no other way can be used to provide the corresponding logical analysis and none of the other works that discussed the meaning of ΔG_{app} has actually evaluated consistently the relevant quantity. This is of course most obvious when the calculations equated ΔG_{app} with the M/TR equilibrium. In this respect we note that even the instructive attempt¹⁹ to simulate the insertion process by a very qualitative CG model could not be used to analyze ΔG_{app} , since the barriers were probably too low (see discussion in ref⁹). Thus, we believe that it is essential to calculate the partition for the different paths considered in our study and this was not been done before.

Continuing along the line of the previous paragraph it is useful to examine a very recent work⁸ that attempted to address the nature of ΔG_{app} . This work, was considered by one of the referees of our paper as a major advance (that presumably makes the kinetic analysis introduced in our works not new nor useful) evaluated by massive microscopic simulations the PMF for the motion from the TR to the membrane, without evaluating the much more challenging barriers for any other key steps, and then tried to determine ΔG_{app} . Here abandoning the assumption of the earlier simulation study^{5,6} (that considered ΔG_{app} as being equal to the M/TR free energy difference) it was attempted to consider implicitly other paths in addition to the TR→M path. Unfortunately, the process of entry to the TR was represented in a phenomenological way by assigning the assumed corresponding rate constant to a parametrically dependent “commitment” time, while the exit to water (“secretion”) was assumed to be irreversible and probably determined by the assumed diffusion constant (which is very problematic⁹). Finally, the membrane insertion process following the first TR to membrane step was considered to be irreversible (again without a clear definition what determine the irreversible motion). Now, since the calculated TR→M PMF yielded an almost barrierless downhill motion for hydrophobic helices, the model simply involved (despite its sophisticated treatment) two competing diffusions paths, one with a small downhill slope (of the calculated PMF) for going from the TR to membrane,

and the other with an undefined PMF of the secretion path and unjustified zero slope (as can be deduced from the fact that no slope is given) and no calculated barrier. The model also involves an arbitrary region where the irreversible part of the insertion step starts. This oversimplified and unjustified model has several serious problems. First, the missing barrier for moving to water (the secretion) and the corresponding missing slope (in case there is no barrier) should reflect the TR/W free energy difference and thus can change the result in any direction. Similarly, assuming an irreversible transfer to water is problematic since we have barrier for this step and since the free energy difference may allow back transfer. More seriously, the calculated time for transfer to water that reflects the assumption of no downhill PMF and no barrier makes the analysis arbitrary. The same problem also exists with regards to the speed of the irreversible step of insertion to the membrane. Thus for example, with zero barrier and fast (depending on the chosen diffusion constant) irreversible insertion to water we will always get ΔG_{app} close to zero. The problem is compounded by the fact that as long as the ribosome is attached to the TR the free diffusion to the membrane is not allowed, as outlined in the analysis of Fig.3.

Obviously a proper analysis requires a clear kinetic scheme, where once the barriers and the free energies are defined and estimated, the time dependence and ΔG_{app} can be deduced (using the energetics and if needed transmission factor corrections) without the need of any sophisticated time dependence calculations. Overall it seems to us that it is impossible to formulate, address and examine the partition between the different phases involved without a corresponding kinetic considerations (such as those introduced in our previous paper⁹ and the present one). Furthermore, having a model that is independent of the free energy difference of the TR/W transition or the relevant barrier, make the results very arbitrary and hard to analyze.

Here reproducing the fact that ΔG_{app} is much smaller than the corresponding microscopic free energy estimates does not actually prove that the actual reason was deduced. That is, any result can be obtained with different rates for the presumed irreversible steps and the real task is to use the microscopic simulations and to get with a well-defined molecular picture the correspondence between ΔG_{app} and the free energies of the model. More specifically, the only point proven in ref⁸ is that with different set of assumed irreversible times it is possible to move from having the segment secreted to water to having all the segment end up in the membrane. In most of the limits the effect of the TR/M initial partition will be secondary and is likely be smaller than the actual W/M free energy difference. Obviously this finding does not prove that the irreversibility assumption is justified since no free energy and barriers have been ever estimated for the final steps in water or for the irreversible motion to the membrane. In fact, we would like to point out that in the limit of irreversible transfer for both the membrane and water, the analysis given in Eq. 8 is likely to be significantly more reliable than that given by the model of ref⁸ as Eq. 9 reflects the free energy of moving both to the membrane and to water. Thus it is significant that Eq. 9 is related to the W/M equilibrium rather than the TR/M difference.

One may try to argue that different barriers and assumptions in our diagram are not based on known facts or are not exact. However, having a clear logical analysis of the possible energetics of different mechanistic steps, is by far more constructive than assuming arbitrarily the meaning of ΔG_{app} and in particular assuming that it is equal to the membrane/TR equilibrium. In fact, our main point is that any actual assertion that the equilibrium between the TR and the membrane determines ΔG_{app} , should be formulated in a way similar to the present analysis, since otherwise such a proposal cannot be verified or falsified. Here obtaining even a very qualitative free energy profile forces one to formulate the problem in a clear way, including accounting for finding a fraction of the system in the W state. We also

like to point out that our conclusion that ΔG_{app} for Arg is not equal to the energy of inserting Arg from water to membrane, will stay valid with any reasonable kinetic scheme.

Finally we like to point out that Eq. 2, that has not been derived prior to the work of ref⁹, represents the equilibrium limit. Thus deviations from this equation can be used (in particular in the more rigorous context of Fig.3) to explore barriers that are responsible for kinetic nonequilibrium steps. However, this cannot be done without a kinetic scheme. In fact, talking on irreversible steps without defining them in a kinetic diagram can only lead to a circular logic.

Acknowledgments

This work was supported by NIH grant GM40283. We thank the University of Southern California's High Performance Computing and Communication Center (HPCC) for computer time.

References

1. Hessa T, Kim H, Bihlmaier K, Lundin C, Boekel J, Andersson H, Nilsson I, White SH, Heijne G von, von Heijne G. Recognition of Transmembrane Helices by the Endoplasmic Reticulum Translocon. *Nature*. 2005; 433:377–381. [PubMed: 15674282]
2. Dorairaj S, Allen TW. On the Thermodynamic Stability of a Charged Arginine Side Chain in a Transmembrane Helix. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:4943–4948. [PubMed: 17360368]
3. Johansson ACV, Lindahl E. Protein Contents in Biological Membranes Can Explain Abnormal Solvation of Charged and Polar Residues. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:15684–15689. [PubMed: 19805218]
4. Rychkova A, Vicatos S, Warshel A. On the Energetics of Translocon-assisted Insertion of Charged Transmembrane Helices into Membranes. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:17598–17603. [PubMed: 20876127]
5. Schow EV, Freites JA, Cheng P, Bernsel A, von Heijne G, White SH, Tobias DJ. Arginine in Membranes: The Connection Between Molecular Dynamics Simulations and Translocon-mediated Insertion Experiments. *The Journal of membrane biology*. 2011; 239:35–48. [PubMed: 21127848]
6. Gumbart J, Chipot C, Schulten K. Free-energy Cost for Translocon-assisted Insertion of Membrane Proteins. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:3596–3601. [PubMed: 21317362]
7. Zhang B, Miller TF. Hydrophobically Stabilized Open State for the Lateral Gate of the Sec Translocon. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:5399–5404. [PubMed: 20203009]
8. Gumbart J, Teo I, Roux B, Schulten K. Reconciling the Roles of Kinetic and Thermodynamic Factors in Membrane-protein Insertion. *Journal of the American Chemical Society*. 2013; 135:2291–2297. [PubMed: 23298280]
9. Rychkova A, Warshel A. Exploring the Nature of the Translocon-assisted Protein Insertion. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:495–500. [PubMed: 23269832]
10. Sääf A, Wallin E, von Heijne G. Stop-transfer Function of Pseudo-random Amino Acid Segments During Translocation Across Prokaryotic and Eukaryotic Membranes. *European journal of biochemistry / FEBS*. 1998; 251:821–829. [PubMed: 9490057]
11. Ulmschneider JP, Andersson M, Ulmschneider MB. Determining Peptide Partitioning Properties via Computer Simulation. *The Journal of membrane biology*. 2011; 239:15–26. [PubMed: 21107546]
12. Warshel, A. *Computer Modeling of Chemical Reactions in Enzymes and Solutions*. Vol. Vol. 1991. Wiley; 1991. p. 236

13. Messer B, Roca M, Chu ZT, Vicatos S, Kilshtain AV, Warshel A. Multiscale Simulations of Protein Landscapes: Using Coarse-grained Models as Reference Potentials to Full Explicit Models. *Proteins-Structure Function and Bioinformatics*. 2010; 78:1212–1227.
14. Vicatos S, Roca M, Warshel A. Effective Approach for Calculations of Absolute Stability of Proteins Using Focused Dielectric Constants. *Proteins-Structure Function and Bioinformatics*. 2009; 77:670–684.
15. Warshel, AMolaris-XG. Theoretical Background and Practical Examples. http://laetro.usc.edu/programs/doc/theory_molaris_9.11.pdf.
16. MacCallum JL, Bennett WFD, Tieleman DP. Partitioning of Amino Acid Side Chains into Lipid Bilayers: Results from Computer Simulations and Comparison to Experiment. *The Journal of general physiology*. 2007; 129:371–377. [PubMed: 17438118]
17. Rychkova A, Mukherjee S, Bora RP, Warshel A. Simulating the Pulling of Stalled Elongated Peptide from the Ribosome by the Translocon. *Proceedings of the National Academy of Sciences*. 2013; 110:10195–10200.
18. Junne T, Kocik L, Spiess M. The Hydrophobic Core of the Sec61 Translocon Defines the Hydrophobicity Threshold for Membrane Integration. *Molecular Biology of the Cell*. 2010; 21:1662–1670. [PubMed: 20357000]
19. Zhang B, Miller TF. Long-timescale Dynamics and Regulation of Sec-facilitated Protein Translocation. *Cell Reports*. 2012; 2:927–937. [PubMed: 23084746]

Fig. 1.

A kinetic scheme for the analysis of ΔG_{app} . The states are labelled as follows: A – initial state prior the H-segment insertion into the TR, TR – H-segment inside the TR, W – H-segment fully translocated into the exoplasm, M – H-segment inserted into the membrane. The figure considers the TR, the membrane, the engineered leader peptidase protein with three transmembrane domains and the ribosome. It is assumed that the ribosome is detached before the glycosylation step (g designated the glycosylation) but other options are feasible. States W_g and M_g represent the final cases after the protein synthesis has been terminated. The glycosylated sites are marked with the stars. As discussed in the text the figure represents the assumption that the states that are grouped in W_g are formed at a similar rate as the state M_g . A more rigorous analysis is provided in Fig.3.

Fig. 2.
A schematic free energy profile for moving the H-segment inside the TR, into the exoplasm and into the membrane.

Fig. 3.

A more complete kinetic diagram, which describes feasible paths. The diagram considers a set of reasonable but not unique or certain barriers (written in kcal/mol above the arrows) and energies (written in parentheses near each state). g designated glycosylation and Δ designates the contribution from the peptide synthesis step or from other possible vectorial events. The barriers for the steps where the peptide is pushed out the ribosome is taken as 18 kcal/mol reflecting the barrier of the chemical step and the number of peptide groups and the corresponding observed rate⁹. The first two helices described in Fig. 2 are not drawn for simplicity and they are considered as being in the membrane as in Fig. 2.

Fig. 4.

The change in the insertion profile for moving a cluster of 3 Leu from position 3 to position 14 of the 19 residue long Ala H-segment. The calculations are done by the same CG approach used in ref⁹. Note that the energies of the M and W states are not known even for hydrophobic helices (as indicated by the arrows) and discussed in ref⁹.

Table 1

Estimating the relative apparent free energies for different residues^(a)

Residue	ΔG_{TR}	ΔG_W	ΔG_M	$\Delta\Delta G_{W \rightarrow M}(H)$	$\Delta\Delta G_{W \rightarrow TR}(H)$	$\Delta\Delta G_{W \rightarrow M}(Leu \rightarrow i)$	$\Delta\Delta G_{W \rightarrow TR}(Leu \rightarrow i)$	$\Delta\Delta G_{app}^{obs}(Leu \rightarrow i)$
Leu	-2.73	-0.54	-19.13+ δ	-18.59+ δ	-2.18			
Arg	19.26	1.39	0.02+ δ	-1.37+ δ	17.87	17.22	20.05	3.13
Glu	13.95	-0.36	-2.34+ δ	-1.98+ δ	14.31	16.61	16.49	3.23
GluH	-0.25	0.07	-16.14+ δ	-16.21+ δ	-0.32	2.38	1.86	3.23
Ile	-1.84	-0.29	-18.16+ δ	-17.86+ δ	-1.54	0.73	0.64	-0.05
Ala	-0.82	-0.14	-17.03+ δ	-16.89+ δ	-0.67	1.70	1.51	0.66

^a Energies are in kilocalories per mole. The table summarizes the results of our CG procedure that was used to obtain the free energy of the TR-H-segment system with different residues placed in the center of the segment⁹. The sequence of the simulated H-segment is: AAAALALXLALALAAAA, where the name of the central residue X is listed in the first column. ΔG_{TR} , ΔG_W , and ΔG_M are the free energies of the TR-H-segment system with the segment placed inside the TR, in water and in the membrane, respectively. These values were used to calculate the relative free energy of transfer from water to membrane ($\Delta\Delta G_{W \rightarrow M}$) and from water to TR ($\Delta\Delta G_{W \rightarrow TR}$) for each amino acid (designated with *i*) with respect to Leu as follows: $\Delta\Delta G_{W \rightarrow M}(Leu \rightarrow i) = \Delta\Delta G_{W \rightarrow M}(H) - \Delta\Delta G_{W \rightarrow M}(H_{Leu})$ and $\Delta\Delta G_{W \rightarrow TR}(Leu \rightarrow i) = \Delta\Delta G_{W \rightarrow TR}(H) - \Delta\Delta G_{W \rightarrow TR}(H_{Leu})$. $\Delta\Delta G_{app}^{obs}(Leu \rightarrow i)$ is the observed relative free energy calculated from the hydrophobicity scale^{1, 8} designates the uncertainty in the membrane insertion energy, which reflects in part the energy of the H helix in the membrane/water interface and the current uncertainty in the actual value (see ref⁹). The table includes the also the result for a protonated Glu (GluH) which is the more likely situation