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Synonymous Mutations and Ribosome Stalling Can Lead to Altered Folding Pathways and Distinct Minima

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Abstract

How can we understand a case where a given amino acid sequence folds into structurally and functionally distinct molecules? Synonymous single-nucleotide polymorphisms (SNPs) in the *multidrug resistance* 1 (*MDR*1 or *ABCB1*) gene involving frequent to rare codon substitutions lead to identical protein sequences. Remarkably these alternative sequences give a protein product with similar but different structures and functions. Here we propose that long-enough ribosomal pause time-scales may lead to alternate folding pathways and distinct minima on the folding free energy surface. While the conformational and functional differences between the native and alternate states may be minor, the *MDR*1 case illustrates that the barriers may nevertheless constitute sufficiently high hurdles in physiological time-scales, leading to kinetically trapped states with altered structures and functions. Different folding pathways leading to *conformationally-similar* trapped states may be due to swapping of (fairly symmetric) segments. Domain swapping is more likely in the no-pause case where the chain elongates and folds simulaneously; on the other hand, sufficiently long pause times between such segments may be expected to lessen the chances of swapping events. Here, we review the literature in this light.

Keywords

Synonymous mutations; protein folding; kinetics; multidrug resistance gene; ABC transporter; P-gp

Recently, Kimchi-Sarfaty et al. observed that synonymous single-nucleotide polymorphisms (SNPs) which retain the amino acid sequence can nevertheless result in a protein with an altered structure and function[1]. Synonymous SNPs are silent mutations, which if occurring in protein coding regions, involve nucleotide substitutions still coding for the same amino acid. Since the genetic code is degenerate, many types of such SNPs can be found. Hence, when examined from the standpoint of the stationary amino acid sequence, no change is observed. Yet, in the case of the *Multidrug Resistance* 1 (*MDR*1) gene, the paper reports that despite the identical protein sequence, certain combinations of synonymous SNPs involving frequent to rare codon

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substitutions appear to alter the transport substrate binding site shape and substrate specificity of the gene product, P-glycoprotein (P-gp). Rare codons may result in ribosome stalling[1,2], either due to a lower concentration of cognate tRNAs or an alteration of the RNA structure. Translational stalling enforces sequential folding pathways which may lead to different distinct minima in the folding free energy landscape. The observation that some inhibitors bind to one of P-gp conformational states but not the other; that single residue substitutions may dramatically change substrate specificity[2]; and that the P-gp recognizes hundreds of substrates and inhibitors in a large, pleomorphic substrate/inhibitor interaction site, suggest that the conformational change between the P-gp states is limited. Which of the two states is the "native" and which is the "mutant" is unclear: the haplotype (1236C>T/2677T>A/3435C>T) described in the study by Kimchi-Sarfaty et al[1] is fully functional as a drug transporter, approaching 50% in certain ethnic populations, thus suggesting some substrate-selective advantage. The SNPs that constitute the haplotype have been the subject of many studies and the 3435C>T (at exon 26) is the most commonly implicated in the diverse responses of patients towards a long list of P-gp substrates. These polymorphisms are risk factors for several diseases[3–6] and may affect the progression of others[7–10]. In addition, the pharmacokinetics of several drugs such as cyclosporine A[11], nelfinavir[12], fexofenadine[13], and digoxin[14] are affected by these SNPs. This is not surprising because numerous mutational studies of P-gp have demonstrated that point mutations in mammalian P-gps affect substrate specificity (see Table 1 in Ambudkar et al[15] for an extensive list).

The case of the Multiple Drug Resistance 1 (MDR1) gene

The *Multiple Drug Resistance* 1 gene product P-gp, an ATP-binding cassette (ABC) transporter, is an ATP-driven efflux pump for many substrate hydrophobic drugs that enter the cell via diffusion through the membrane[2]. Consequently, it is hypothesized to play a major role in multidrug resistance during chemotherapy in metastatic cancers. Haplotype polymorphisms coded by the double or triple combinations of mutations (C1236T-G2677T; C1236T-C3435T; G2677T-C3435T; and C1236T-G2677T-C3435T) were less effective against cyclosporine A and verapamil inhibitors, while no change was observed in the transport of a range of substrates for the single polymorphisms of C1236T, C3435T and G2677T[1]. Thus, it is not the nonsynonymous polymorphism at G2677T that is responsible for the altered functional inhibition of P-gp, but the combination of C3435T, a synonymous polymorphism, with either C1236T, C2677T or the C1236T/C2677T haplotype that results in altered specificity. The altered transport implicated a structural change. The binding to conformation-sensitive monoclonal antibodies (UIC2) and trypsin digestion patterns supported such a conclusion. It is also unlikely that the observed altered specificity could be explained by differences in glycosylation of the wild type and haplotype. P-gps which were expressed in mammalian cells and immunoblots show that the wild type and haplotype have identical molecular mass. Further, previous studies have demonstrated that glycosylation does not affect the function of P-gp[16]. Thus the altered haplotype function is not likely to be due to altered glycosylation. Further, (i) cyclosporine and verapamil inhibition differences between the wild type and the C1236T-G2677T-C3435T haplotype increased with the mutant DNA concentration, suggesting a relationship to mRNA levels; (ii) there were no rapamycin inhibition differences between the two species; and (iii) the SNP, although silent, involved frequent to rare codon substitutions.

There is substantial evidence that P-gp exists and functions as a monomer[17]. Currently no structure exists for the P-gp transporter. However, structures of bacterial MDR proteins and their cognate regulators provide a wealth of molecular detail about how these proteins recognize multiple substrates. For example, structures of QacR, the multidrug binding protein that represses the transcription of QacA have been solved individually, with six substrates and with two drugs bound simultaneously[18–20]. These results are broadly consistent with the

structure of the *E.coli* AcrB pump solved with four different substrates[21]. All these structures show the use of a single large cavity comprising a "drug-binding pocket" where individual "drug-binding sites" are generated by subtle alterations in the accessibility of different subset (s) of residues for drug-binding. What these studies clearly demonstrate is that MDR proteins can alter substrate specificity fairly easily and that even subtle changes in the conformation of the drug binding pocket can result in changes in substrate specificity. Moreover, such changes can be measured using several well established assays[22].

Two types of protein folding scenarios to obtain alternate conformations

In both P-gp and amyloids[23–25], under physiological conditions some fraction of the molecules are in an alternate conformation. However, there the similarity ends: In the amyloid case, via conformational selection[24], monomers *in this conformation* may assemble to create the typical cross-β structure[26]. If the assembly is below a certain seed size (at least 4 monomers) the molecules will dynamically associate and disassociate; as the seed grows, stability increases. This kinetic behavior is characteristic of polymerization reactions, suggesting analogy with a nucleated polymerization mechanism[27; 28]. Seed formation constitutes the aggregation bottle-neck. Fibril extension occurs on a much faster time scale than seed formation. Binding and amyloid growth will shift the equilibrium toward this misfolded conformational state[24]. In contrast, in the second P-gp scenario, the altered state relates to protein synthesis and ribosome pausing effects. Depending on the pause time scales, pause sites location, and the protein sequence, such events can lead to alternate folding pathways.

Consequently, there are two types of scenarios: the first involves a transition from the folded to the misfolded and aggregated state[29]; the second relates to a protein folding pathway. The co-translational folding pathway with altered kinetics and intermediate states[30–32], can lead to functionally-distinct proteins. For both cases, the altered conformations need not involve the entire molecules. For amyloids, a local segment may adopt an extended state, with the rest of the structure retaining a native-like state. For the ribosome stalling case, the downstream chain might fold into its "native" conformation. Whether such kinetically-controlled scenarios via rare codon usage are used in nature to regulate function is unclear. As we argue below, the chances of evolution adopting a kinetic control as a regulatory mechanism to obtain *globally* different conformations for a given sequence are fairly low due to the lack of robustness and the risk involved. On the other hand, a *limited* change, with the conformations lying nearby on a rugged folding landscape funnel bottom is likely, allowing them to bind to a broader range of ligands.

Thus, the folded to misfolded transition and aggregation which are observed in neurodegenerative diseases is reflected in a change in the protein folding free energy landscape (Figure 1A). On the other hand, in ribosome stalling cases the landscape is unchanged; instead, different folding pathways may lead to distinct minima and alternate (functional or diseased) conformations (Figure 1B).

Co-translational (sequential) folding

In vivo, evolution via natural selection ensures that the newly synthesized polypeptide chain always folds spontaneously into a native functional conformation with or without help from molecular chaperones. *In vitro*, a small single domain protein with only one hydrophobic folding unit is always able to fold and unfold reversibly[33]. These two observations suggest that the three dimensional fold of a protein is determined entirely by its amino acid sequence. In a sequential folding mechanism, contiguous fragments in the 1D chain interact in the 3D fold, significantly reducing the possibility of misfolding[34–36]. On the other hand, in the more "complex" protein folds, substantial contacts between non-sequential pieces of the chain are observed. Sequential folding is advantageous[36]: since it is kinetically more favorable, it is

expected to be faster. Chain linkage contributes to bringing mutually stabilizing consecutive building blocks together more frequently than segments which are sequentially far. In terms of the folding funnel free energy landscape theory[37; 38], the folding process should encounter fewer rough bumps on the free energy surface in a sequential folding case. This implies that in a sequential folding mechanism the likelihood of misfolding is greatly reduced[36; 39].

It is widely believed that with few exceptions, the native conformations of proteins at physiological temperatures are at their global free energy minima. If this is correct, then misfolding may occur as kinetically trapped alternate conformations, either due to mutations or to changes in folding conditions. Evolution lessened the chances of misfolding by preserving folds that are not only at their thermodynamic global minimum, but are also kinetically favorable. Still, kinetic trapping events may be essential for particular functions such as in the remarkable Serpin protease family[40] case. In protein folding, the strictly sequential interactions between energetically favorable segments fulfill perfectly the requirements for reaching the global minimum without encountering high barriers; and ribosome pausing enforces a sequential folding pathway.

Ribosome pausing effects and folding kinetics

While considerable work focused on protein folding kinetics, uneven translation rates, particularly stalling at given sites over relevant time scales during protein synthesis, have not been thoroughly explored. *In vivo*, folding kinetics relate to two factors: (i) translation rate and evenness as a function of the codon sequence and local mRNA stability, i.e. the mRNA sequence; and (ii) folding rates as determined by protein structural motifs, which under equilibrium conditions are a function of the protein sequence. For the first, (i) *in vivo* translation rates may be affected by the ribosome pause time scales; the longer the pause, the more likely an altered kinetic scenario. Stalling may result from synonymous frequent to rare codon substitutions[41–45] due to lower tRNA populations[46] and slower tRNA recharging[42]; the longer the stretches of predominantly rare codons the longer the pause; or due to local stable mRNA structure[47; 48], or codon/anti-codon base pairing[49]. Since only a certain fraction of the ribosomes will stall, we expect both native and altered conformational states. For the second, (ii) a protein is likely to fold slowly if its folding landscape has at least two minima; on the other hand, it is likely to be a fast folder if it has an all-α fold[50]; undergoes a sequential folding process; and has a low contact order[51] (i.e. where sequentially-close residues are in van der Waals contact) which sequential-folders usually do. Many of the fast folders are small helical protein subdomains[50]. The folding free energy landscape of fast folders is not expected to have high barriers. Under such circumstances, folding rates relate to energy landscape roughness. β-structures fold with slower kinetics, since β-sheets have rougher energy landscape as compared to α-helices, however, β-proteins folding rate measurements are scant compared to α-proteins. Ultrafast folders are rarely observed to populate the partially folded state, quickly achieving their native state conformations.

Let us assume that there is a single or a stretch of silent substitutions at position S_1 (Figure 2) which leads to stalled ribosomes at this site compared to native folding. S_1 divides the protein into an upstream part *A* and downstream part *B*, where *A* and *B* are protein building blocks [36; 52]. If both *A* and *B* are fast folders, pausing at S_1 is unlikely to lead to altered folding pathways and altered conformations, since both native conformations are obtained rapidly. If both *A* and *B* are slow folders, it is also unlikely, since *B* also needs time to reach its favored state. If *A* is a fast folder and *B* a slow folder, *A* will fold into its native state regardless of whether the ribosome stalls, and the folding of *B* will be unaffected. On the other hand, if *A* is a slow folder, and *B* is a fast folder, the situation is different: the slow folder *A* is likely to have competing conformations, say, A_1 and A_2. Let us assume that on its own A_2 is more stable than the native A_1. Under native conditions, the fast folder *B* may serve as a template to A_1 since

their association is favorable; On the other hand, if the ribosome stalls, by the time *B* is synthesized A_2 is already formed. While in this case stalling leads to different branches in the folding pathway, all are on the native pathway, leading to minor conformational changes. Here we related to single-site stalling events. In the *MDR*1 case two or three simultaneous substitutions are observed to lead to such effects, with likely more complex kinetic scenarios.

The statistics relating to the environment and locations of the rare codons is interesting[39]: Studies of *E. coli* observed that (i) fast folding α-helical sequences tend to have fewer rare codons than the slower folding β-sheets[53] which have higher contact order values[51]; (ii) for the *EgFABP1* fatty acid binding protein[45] removal of the three rare codons in a helix-turn-helix leads to higher aggregation rate *in vivo*, but not *in vitro*; (iii) about 70% of protein domain boundaries in *E. coli* are coded by rare codons[54]; and (iv) there is a correlation between gene length and rare codon frequencies: longer genes tend to have higher fractions of rare codons[55]. Further, while actual kinetic data are scant, the three exceptions point that rare codons have slower translation rates (for example, for the frequent GAA codon, the translation rate is 21.6 codons/second whereas for the rare GAG codon the translation rate is 6.4 codons/second[56]); and, the longer the rare codon stretch the slower the translation rate[57;56;58]. Thus, available data uphold the arguments presented above: fast folders avoid rare codons; longer-range secondary structure motifs have a higher chance of containing them to increase 'correct' folding probabilities; longer sequences which have higher misfolding chances have higher tendencies to have rare codons; and significantly, within these, there is a strong tendency for rare codons to be located between domains, leading to pauses and consequently to higher chances of obtaining favored thermodynamic states. Rare codons enforce an order on the folding of the protein. Thus, it follows that ribosome slowing at certain sites may lead to different folding pathways reaching distinct minima with minor conformational changes.

A role for a template, or an “intramolecular chaperone”?

Since native ultrafast folders are generally subdomains of larger proteins, fast folding domains may assist the folding of large proteins. The recalcitrant proteins substantiate such a scenario, where “assisted-folding” takes place via fusion to a poorly-folding protein[59]. Fast folding (sub)-domains act as templates or “intra-molecular chaperones”[52]. An intramolecular chaperone catalyzes folding, and neither dissociates nor is cleaved. Yet, while intermolecular chaperones catalyze folding by unfolding misfolded conformations or preventing them, an intramolecular chaperone catalyzes folding by binding to, stabilizing and increasing the populations of native conformations of adjacent fragments. One example of an intramolecular chaperone is the amino-terminal pro-region fragments of some proteases, such as bacterial subtilisin, alpha-lytic protease, and aqualysin, and yeast carboxypeptidase Y, which are cleaved after assisting in the folding[60]. Since proregions act as inhibitors covering the enzyme active site, they must be removed for the enzymes to be functional. However, if the fragment is cleaved prior to complete protein synthesis, the chain misfolds. Mixing the cleaved fragment with the remainder of the chain leads to a correctly folded protein[61; 62]. An intramolecular chaperone does not work by binding to an intermediate conformation and thereby inducing it to undergo a conformational change to the native state; rather, it binds to the native state via conformational selection[23–25; 63], and thereby leads to an equilibrium shift[63] which propagates the binding reaction. In our ribosome pausing scenario case, the fragment labeled as *B* (Figure 2) is an intramolecular chaperone, assisting in the folding of *A* via conformational selection of A_1 rather than unfolding A_2. Two-domain spectrin[64] presents a similar cooperative scenario. While mechanistically intramolecular chaperones and proregion segments are similar, the (inhibitory) proregion function leads to a difference: proteins folding with proregion assistance are under kinetic control; on the other hand, proteins with uncleaved intramolecular chaperone are under thermodynamic control.

Ribosome stalling and co-translational folding: some examples

Numerous examples illustrate that co-translational folding reduces the chance of misfolding, and that faster protein synthesis rates increase the chance of 'incorrectly' folded proteins. When protein synthesis rate is fast, the entire chain is already synthesized when the protein folds. An early example relates to ovalbumin[65]. Renatured ovalbumin (OAR) was separated from the native form (OAN), suggesting a 'preferred' pathway leading to the native state. Going back to our scenarios (Figure 1B, Figure 2), since in renaturation the entire sequence is already synthesized, the refolding of the OAR state resembles the case with no ribosomal pause at S_1 versus the likely sequential folding of OAN. Replacement of 16 consecutive rare by frequent codons in *E. coli chloramphenicol acetyltransferase (CAT)* gene lead to protein synthesis rate acceleration and 20% specific activity reduction[44]. Hence, as in OAR, conformations are caught in energy minima in the rugged funnel bottom. In globin[66], a ribosome-bound nascent 86-residues fragment already attains its native favored state, implicating co-translational folding. Silent mutation effects have also been observed: the *TRP3* gene encodes a bifunctional protein, with anthranilate synthase II and indoglycerol-phosphate synthase activities[67]. Replacement of ten consecutive rare by frequent codons in the second region led to reduced activity[43;67], suggesting conformational change. Synonymous rare-to-frequent codon substitutions were introduced into the *EgFABP1* (*Echinococcus granulosus* fatty acid binding protein) gene[45] leading to *in vivo* aggregation. Concentration is critical: in low concentration, even fast synthesis is likely to yield proteins in their favored states; in contrast, if the concentration is high, the chances of aggregation are higher.

While there are no direct data relating to P-gp folding, another ABC transporter, the cystic fibrosis transmembrane conductance regulator (CFTR), was shown to fold co-translationally[68]. With the C-terminus truncated, the domains still form well-defined structures. CFTR folds during translation, translocation and membrane insertion, implying slow co-translational folding. Deletion of Phe508 in the nucleotide binding domain 1 (NBD1) causes limited alteration in NBD1[69]; however it disrupts the folding of NBD2. At the earliest biosynthetic stages, wild-type and ΔPhe508 CFTR are susceptible to degradation (all ΔPhe508 CFTR and 45–80% of wild type CFTR), possibly indicating that they still have not attained their native conformation[70,71]. The results of Varga et al.[72] suggest a small conformational change.

The 'native' and 'altered' conformational states

Above we focused on a case where a slowed translation could lead to a protein product with an *altered conformation*, whereas the examples we provided address cases where accelerated translation leads to aggregation or *reduced activity*. Are these two scenarios different sides of the same coin?

When studying an enzyme the distinction between 'correctly folded' and 'mis-folded' is relatively straightforward: as a general rule the binding pocket is specific for a given substrate. On the other hand, in the case of promiscuous transporters such as those belonging to the ABC family this distinction is difficult to make. The most detailed structural view comes from the few structures of regulatory proteins of bacterial multidrug-transporters. These studies show a large drug binding pocket that can accommodate several substrates and different drugs utilize a different subset of residues. The interactions can be quite complex and often counterintuitive. To give an example, in QacR, the repressor of qacA, although different drugs utilize a different subset of residues, most drugs contact one or more of the four glutamate residues[19; 73; 74]. The events that follow initiation of translation, protein translocation, folding, and degradation, are facilitated by molecular chaperones. Soluble proteins are translocated into the ER lumen, whereas transmembrane proteins are cotranslationally integrated into the lipid bilayer[75]. The

degradation of misfolded proteins is as important as the translocation and folding of proteins and in membrane proteins this involves an additional level of complexity, that is, the retro-translocation of proteins from the endoplasmic reticulum into the cytosol[76]. If it is not degraded, it can lead to disease. Thus, the misfolding of a mutant form of the ABC transporter, CFTR, is responsible for cystic fibrosis. The chaperones and cofactors involved in the folding and degradation of this protein have been studied in considerable detail[77]. Given the complexity of this system it can be argued that in the case of the haplotype, a 'mis-folded' protein escapes the quality control machinery. We, on the other hand would suggest that the haplotype generates a protein that is not 'mis-folded' but an alternative conformation. Again, in this situation (relatively unique to P-gp) it is difficult to define the native conformation and thus what is mis-folding. It is probably precisely for this reason that the so-called mis-folded protein escapes the quality control machinery. We emphasize that the levels of wild type protein and haplotype at the cell surface are comparable[1]; if the protein synthesized from the haplotype were mis-folded and ‘escaped’ the quality control machinery of the cell only a fraction of the mis-folded protein would be expressed at the cell surface. That is, if it were misfolded, it would be translocated and degraded leading to lower concentration.

The origin of minor conformational changes: domain swapping?

It is generally accepted that the protein folding free energy landscape has a funnel-like shape. Visualizing the protein folding process as a combinatorial assembly of building blocks[78,79] can describe all major folding pathways. The order in which the building blocks fold toward the final native conformation of the protein constitutes a particular folding pathway. If the surface at the bottom of the funnel is smooth, there is only one native conformation and all folding pathways lead to it; if the funnel bottom is rugged, different pathways may lead to distinct structures separated by not-so-easy to surmount barriers (Figure 2). This is the origin of minor conformational changes for (i) sequential *in vivo* folding versus folding ↔ unfolding experiments *in vitro* and (ii) a ribosome stalling mechanism. Under such circumstances, preferred folding pathways are altered leading to minor conformational changes.

In co-translational protein folding the energy landscapes vary. Co-translational folding scenarios consider events as the chain elongates; thus increasing the formal number of conformational degrees of freedom. It is intriguing to consider which type of phenomena would retain similar functional sites and functional mechanisms as observed in the P-gp, while at the same time would be due to altered pathways; that is, which phenomena would lead to two similar conformations which are separated by physiologically sufficiently high barriers which will not be easily crossed. One such phenomenon is *intramolecular domain swapping*. In a domain swapping mechanism in homodimers, one segment in one monomer is replaced by an equivalent segment from the identical chain in the dimer[80; 81]. For some proteins, such as the bovine seminal ribonuclease (BS-RNase) it was proposed that both conformations co-exist: one with swapped N-terminal segment and the other without segment swapping[82; 83]. It was further suggested that the swapping may have arisen at a later stage in evolution due to functional needs.

In our case, it would be reasonable to consider a scenario where a segment-swapping occurred in the faster folder “native” conformer, unlike in the slower “mutant” folder. A segment-swapping event would lead to similar conformations with the only difference being a swapped segment; yet there would be difficult-to-cross barriers in biological time scales to alter the locations of these segments. In the bacterial ABC transporter homodimer[84] it is reasonable to assume that under appropriate conditions, such as high concentration, a swapping of the last 44 residues at the C-terminal segment may occur between the two monomers (residues 535–578; with the structure consisting of a two α-helices; a β-turn and a third α-helix). Unfortunately, there is no crystal structure for the human P-gp, a monomer. Based on the

bacterial structure, the two (largely symmetric) segments constituting the nucleotide binding domain (NBD) in the P-gp monomer can be swapping candidates. Such a swapping mechanism predicts that ribosome pausing occurring in the first transmembrane domain or in the first NBD would have no effect on the domain swapping events; on the other hand, 'long-enough' pauses following the translation of the first NBD segment and prior to the second can lead to non-swapped conformations. Thus, the "native" may consist of either an ensemble of non-swapped and swapped species, or only swapped; the mutation-induced pausing will shift the landscape toward the non-swapped population. Such pauses can prevent domain swapping events which are observed in fast "native" folder. This may explain the difficulty in crystallizing the mixed population of "native". Domain swapping effectively leads to kinetically trapped conformations.

Conclusions: minima at the bottom of the funnel can have physiologically sufficiently high barriers leading to distinct conformations

Proteins are critical for all biological processes. To ensure proper function, they need to have the 'correct' structure in the 'right' population, with a 'favored' energy landscape. All the necessary information is encoded in the sequence; yet, it is unclear how nature has engineered the protein folding mechanism and control. Recently, frequent to rare synonymous codon substitutions in the *MDR*1 gene were implicated in at least two distinct P-gp conformations with altered functions. Rare codons lead to stalled ribosomes; hence, depending on the pause time scales, the upstream parts of the protein can fold prior to the downstream synthesis, affecting folding pathway scenarios. P-gp is a transporter protein. Unfortunately, not much is known about the folding of membrane proteins as compared to globular proteins, and even less is known about their complex kinetics which are likely to be affected by the interactions with the bilayer[85].

The case of the *MDR*1 gene provides a nice example of a mechanism that is likely to be used by nature to expand the functional repertoire. *MDR*1 illustrates that stalling can lead to altered folding pathways, culminating in different minima in the folding funnel bottom. Yet, perhaps the most interesting point here is that while the structures are likely to possess only a limited conformational change and the free energy difference between them is likely to be small, the barriers are sufficiently high on physiological time scales, leading to the distinct conformational and functional states. Actually, in retrospect, this is not surprising: crystallization takes time due to the need to await identical conformers, in addition the inherent improbability of bringing 3, 4, or 5 monomers together to form a viable nucleus. The surface is rough, and time is required to surmount the barriers. For the *MDR*1 gene product, the fact that the conformers are distinct is observed from the different binding patterns of the Abs, the trypsin digestion and the difference in the extent of inhibition by cyclosporine A and verapamil. Yet, it is also clear that the extent of the conformational change is not too large: native and polymorphic P-gps are inhibited to similar extents by rapamycin.

Is nature using such a mechanism for function? We believe that this is the case *for small conformational changes*. P-gp is known to bind hundreds of substrates, and this mechanism may already operate there. While neither the structure of P-gp nor the locations of the SNP in the tertiary structure are unknown, it is rational to assume that even a small change in the membrane-buried part of the protein can lead to a large change in the extra-membranous conformation, affecting the affinity of the mutant to the ligands. A conformational change in the membrane is expected to be small, since the membrane will not support a large change. There are numerous proteins used for multiple functions, in particular those with a range of ligands. The conformationally flexible *hub* proteins are likely to be a yet another example. Hubs are proteins with shared binding sites[86] which are critical in cellular networks, mediating numerous functions. They are frequently flexible, marginally stable or disordered on their own.

Yet, when crystallized in complex with partner proteins, they still assume a similar overall shape. Even in the binding site, the conformational difference can be remarkably small. On the other hand, a *large* conformational change under kinetic control is not a robust mechanism. Barrier heights are sensitive to environmental conditions, and over time the proteins will flip into their thermodynamically most stable states. While there are proteins under kinetic control, like the proteases discussed above, such cases appear to be rare and designed for a specific function. The sensitivity of kinetic control to the environment is also indicated by the absence of dual-conformation cases in the Protein Data Bank and the lack of such observed cases of *globally* different conformations in folding-unfolding experiments. Protein sequences appear to be selected by evolution to avoid such traps.

We conclude that different folding pathways may lead to distinct minima at the bottom of the protein folding funnel however with a small conformational change; nevertheless, the barrier heights, even if small, may be physiologically sufficiently high to lead to a new type of function or a conformational folding-pathway alteration. Similar mechanisms may also operate for proteins with *shared binding sites*; this would allow a smaller number of genes to perform an increasing number of functions. Distinct minima with small conformational changes may explain the more centralized nature of the cellular network and how central regulatory proteins are able to bind an astonishingly large number of different partners.

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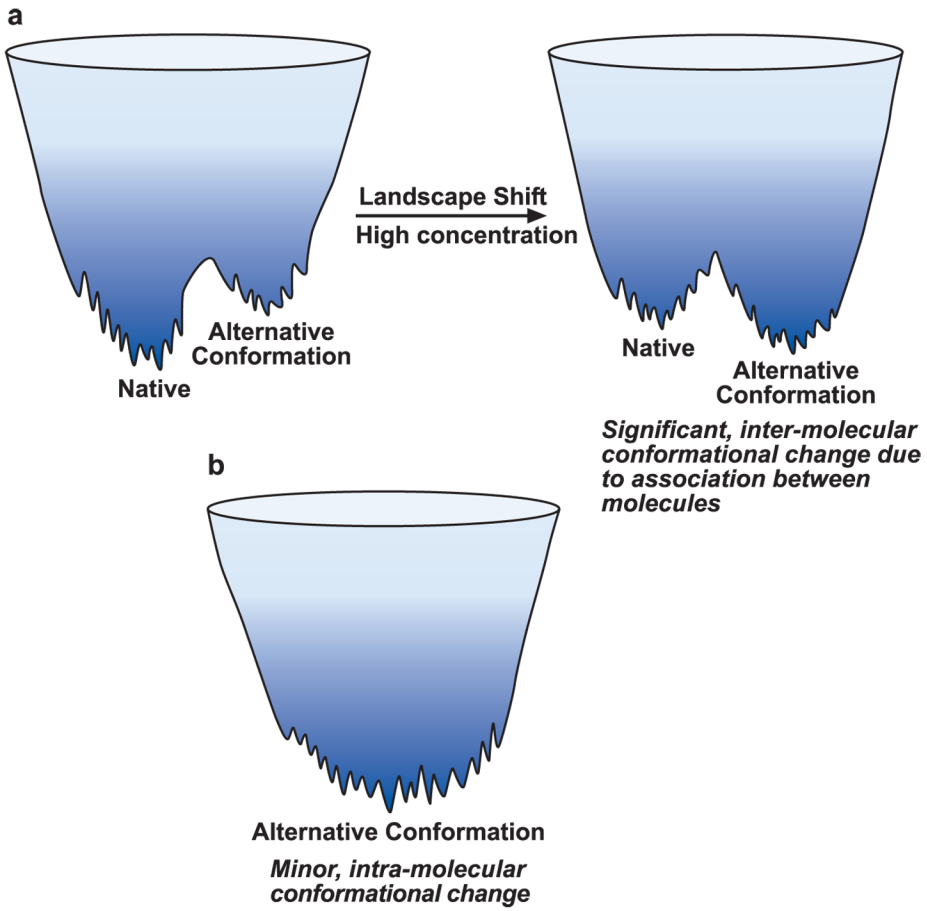

Figure 1.

Simplified folding free energy landscape to illustrate two types of protein folding pattern scenarios. At the single molecule level, the native protein conformation is more favorable thermodynamically than the altered conformation due to synonymous mutations (Figure 1A). However, the folding free energy shifts toward favoring the haplotype conformation at high concentration with a portion of the conformation changed significantly due to inter-molecular association. The sizable barrier in Figure 1A reflects the involvement of a significant conformational change. Figure 1B illustrates the second type of folding pattern. At the bottom of the folding funnel, the landscape is rugged with many local minima, each representing a similar but distinct core structure. The intermediate barrier in the Figure 1B is to emphasize that there is only a minor conformational change when moving from one local minimum to the other. Unlike the first (amyloid) folding pattern, here the folding free energy landscape will not change since it does not involve inter-molecular interactions. The final folded conformation is mainly controlled by folding kinetics: a different folding pathway leads to a different conformation.

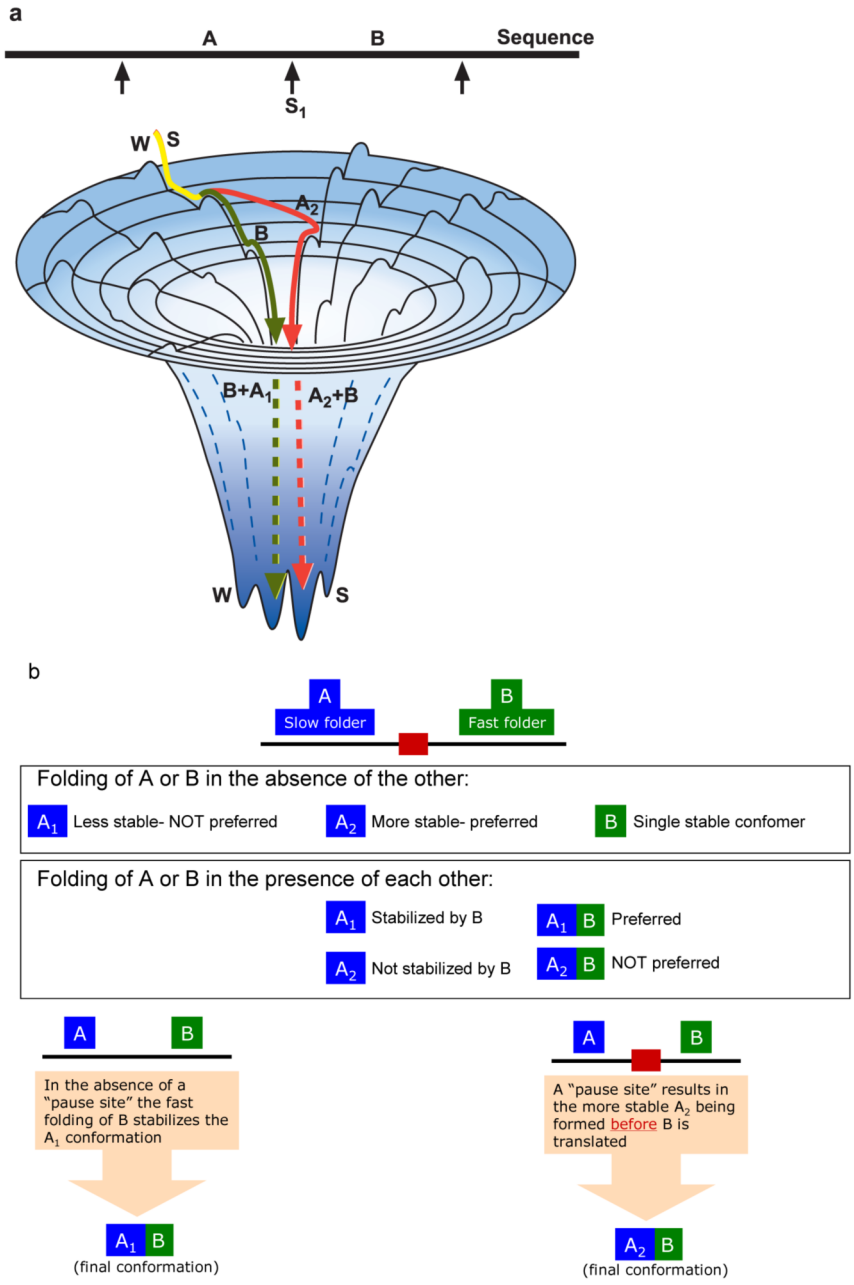

Figure 2.

A simple scheme to illustrate the origin of a minor conformational change via a ribosome stalling effect. Along a sequence, say at an arbitrary position S_1, fragment *A* preceding S_1 is a slow folder and the fragment *B* following S_1 folds faster than fragment *A*. Also, fragment *A* has two competing conformations, A_1 and A_2 with A_2 more stable than A_1 by itself but A_1 becomes more favorable in the presence of fragment *B*. Let us assume that the folding of the nascent chain is independent. Then the folding landscape will be exactly the same for both the wild sequence (W) and the sequence (S) with a synonymous mutation at S_1 since they have the same amino acid sequence. The co-translational folding pathway is expected to be identical up to the S_1 position. In Figure 2A the folding pathways are drawn as step-by-step arrows on

the simplified folding funnel surface. Without a pause at S_1, fragment *B* folds before fragment *A*; then fragment *A* folds on fragment *B* with an A_1 conformation. On the other hand, for the synonymous mutation at S_1 case, the pause enables A_2 to fold first and fragment B follows. The folding branches due to a pause in a sequential folding eventually lead to the bottom of funnel with minor conformational change between them. Figure 2B provides a diagram of the two folding scenarios.