Protein folding in high-dimensional spaces: hypergutters and the role of non-native interactions

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Abstract

We explore the consequences of very high dimensionality in the dynamical landscape of protein folding. Consideration of both typical range of stabilising interactions, and folding rates themselves, leads to a model of the energy hypersurface that is characterised by the structure of diffusive “hypergutters” as well as the familiar “funnels”. Several general predictions result: (1) intermediate subspaces of configurations will always be visited; (2) specific but non-native interactions are important in stabilising these low-dimensional diffusive searches on the folding pathway; (3) sequential barriers will commonly be found, even in “two-state”proteins; (4) very early times will show characteristic departures from single-exponential kinetics; (5) contributions of non-native interactions to Φ-values are calculable, and may be significant. The example of a three-helix bundle is treated in more detail as an illustration. The model also shows that high-dimensional structures provide conceptual relations between the “folding funnel”, “diffusion-collision”, “nucleation-condensation” and “topomer search” models of protein folding. It suggests that kinetic strategies for fast folding may be encoded rather generally in non-native, rather than native interactions. The predictions are related to very recent findings in experiment and simulation.

1 Introduction

The current conceptual map of protein folding kinetics is dominated by the coexistence of several apparently distinct approaches. They may be categorised loosely into “energy landscape” (Bryngelson et al., 1995; Onuchik, 1995), “diffusion-collision” (Karplus and Weaver, 1976, 1994), “nucleation-condensation” (Fersht, 2003) and “topomer search”
(Makarov and Plaxco, 2002) models. Each of these has its own way of visualising how the collapse of a random coil to a native globule can ever be accomplished in observable time scales, a problem pointed out long ago (Karplus, 1997). Each has advantages and drawbacks, but it is not clear whether each applies to a restricted subset of real cases, or whether all might have something to say about the folding of any one protein.

The “folding-funnel” picture of the energy landscape has the advantage of visualising both guided folding and the emergence of on-pathway and off-pathway intermediate states (Dinner et al. 2000). Yet it is hard to escape from the deceptive simplicity of low-dimensional projections of folding funnels that appear necessarily in all graphical portrayals of it. In practice of course, the dimensionality of the folding space is enormous. Even small (∼ 100 residue) proteins have a configurational space dimensionality of several hundred (think of the bond angles along the polypeptide main chain alone). In such high-dimensional spaces, qualitatively new features may arise, such as energetically-flat domains that nonetheless are extremely difficult to escape from and so behave as kinetic traps. A second feature is the potential for high cooperativity of structure in several simultaneous dimensions. This corresponds to the existence of narrow gullies in the hypersurface that are hard to find. In more biochemical language these structures might be exemplified by cooperative secondary structure formation alongside native or near-native distant contacts in α-helix bundle proteins (Myers and Oas, 2001), or simultaneous folding and anion binding (Henkel et al. 2001).

The “diffusion-collision” approach, on the other hand, is supported by strong experimental evidence that folding rates are controlled by the rate of diffusion of pieces of the open coil in their search for favourable contacts, rather than a driven collapse along some continuous energy surface (Jacob et al., 1999; Plaxco and Baker, 1998; Goldberg and Baldwin, 1995). Pre-formed units of secondary structure diffuse hydrodynamically and merge. Larger proteins may do this in an increasingly hierarchical way. The importance of diffusive searches is unsurprising, since under biological conditions, all candidates for energetic interactions, including electrostatics, are locally screened to a few angstroms: much smaller than the dimensions over which sections of protein must move to find their native configurations. Put another way, the vast majority of the space covered by the energy landscape must actually be flat (on a scale of $k_B T$) rather than funneled. Simple versions of these models have indeed been able to account rather well for folding rates as a function of secondary structure formation (Myers and Oas, 1999, 2001). However, it is not clear how applicable this approach is to cases in which secondary structure forms within a collapsed globule or cooperatively with it.

An attempt to articulate a range of scenarios in which partial ordering of secondary and tertiary structures mutually enhance a favourable folding pathway has been presented
under the label of “nucleation-condensation” (Dagett and Fersht, 2003). Originally conceived as a kinetic theory in which a nucleus of native structure corresponds to the transition-state for folding, the picture now also encompasses the hierarchical folding routes of the diffusion-collision model.

A challenge faced by all these models is that the most successful search for inherent features of tertiary structure that correlate with folding rates has found that the topological measure of “contact order” is far more closely related than, for example, molecular weight itself in the case of “two-state” folders (Plaxco et al 2000). Rationalisation of this observation has given rise to a third view of the critical pathway of protein folding, the “topomer search” model (Plaxco and Gross 2001). The rate determining step is not the rapid formation of local secondary structure, nor the diffusion of subdomains per se, but the organisation of large pieces of secondary structure into the same topological configuration as the native state, which is thereafter able to form rapidly. This suggests a partition of the folding space into “rapid” dimensions representing the local formation of secondary structure, and “slow” dimensions representing the topomer search. However, a quantitative relation between the topomer search space and contact order is still unclear, since no native contacts are actually required to form at a purely topologically-defined transition state at all (although many are to be expected from the partial ordering at the secondary level at least). Furthermore, information on the effect on folding rate of replacing specific residues via mutation or “Φ-value” analysis (Fersht, 2000) needs to be taken together with correlations of contact order.

These four approaches have one important aspect in common: they all effectively reduce the dimensionality of the search-space by assumption, rather than in a derived way. This is both natural and necessary, since data from kinetic experiments do just the same, but there is a danger in overlooking aspects of folding that rely essentially on the presence of many degrees of freedom. Our aim in this work is to take a fresh look at the issue, embracing many simplifications but on this occasion not that of a low dimension of configurational search space. We find in the next section that quite general conclusions may be drawn about the topology of this search space if the dimensionality is kept high. Some general predictions follow which we work out in more detail in the case of three-helix proteins. The approach will additionally allow us to see how the existing apparently-distinct paradigms for protein folding are related, and suggest places to look for the information content of the “kinetic code” within proteins that encodes the folding search path, as distinct from the native structure itself.
We start with a very simple and abstract model for protein folding, but one that explicitly retains a very large number of degrees of freedom. The total search space is modelled as the interior of a hypersphere of dimension $d$ and radius $R$, and the native (target) state as a small sphere of radius $R_N$ at the origin of the space. The entire configuration of the protein corresponds to a single-point particle executing a random walk in the hypersphere. The ratio of $R$ to $R_N$ describes the typical localisation on folding in the values of a degree of motional freedom. If the degree of freedom is spatial the appropriate scales are the size of a molten globule and the radius of gyration of a denatured protein. If it is angular, then they are the angle of libration of a bond fluctuating in one local minimum as a fraction of $2\pi$. In either case the appropriate order of magnitude estimate is which is $(\frac{R}{R_N}) \simeq 10$. Bicout and Szabo (Bicout and Szabo 2000) introduced this very general framework for discussing flat and funneled landscapes, but then restricted themselves to three-dimensional spaces, a simplification that we shall try to avoid. To explore the timescales of the search for the target space (on which the diffuser will “stick”) we write down the time-dependent diffusion equation for a particle, restricting ourselves to the case of a flat potential at first. The most convenient function to use is the probability density $P(r, t)$, that the system is a radial distance $r$ from the centre of the hypersphere at time $t$, which obeys:

$$\frac{\partial P(r, t)}{\partial t} = D \frac{1}{r^{d-1}} \frac{\partial}{\partial r} r^{d-1} \frac{\partial P(r, t)}{\partial r}$$

supplemented by the absorbing boundary condition $P(R_N, t) = 0$, signifying the stability of the native state. The timescale for the search steps is set by the effective diffusion constant $D$. The mean passage time from the unfolded ensemble to the native state can be calculated by introducing a uniform current $J$ of diffusers (representing a population of folding proteins) on the boundary of the hypersphere at $r = R$, as the other boundary condition, and finding the consequent steady state solution to (1). The mean time to pass from $R$ to $R_N$ over the ensemble of systems is then just the total number of diffusers at steady state normalised by the current, leading to

$$\tau_f = \frac{1}{d(d-2)} \left( \frac{R}{R_N} \right)^{(d-2)} R^2 \frac{1}{D}$$

This expression indicates how very much qualitatively longer the mean search time is in high dimensions ($d > 2$), than the low-dimensional estimation of the characteristic time $\tau \simeq R^2 / D$, which replaces (2) in $d = 1$, and 2. This fundamental time is scaled up by the denatured system size (measured in units of the target size $R_N$) to the power of the number of effective dimensions greater than 2. An analysis of the eigenmode structure of
the problem indicates why this is so: for large $d$ nearly all the diffusers exist in the lowest

eigenfunction of the diffusion operator, that is in turn localised to the exponentially large

surface of the hyperspherical search space. Single-exponential kinetics are also a general

property of such high-$d$ search spaces.

The central result of (2) depends on two key physical assumptions: (1) the dimensionality of the space is of realistic values for protein folding - of the order of a hundred

or more, and (2) the stability of the folded state is governed by local interactions in the

native state only. With these assumptions alone, the model of high dimensional diffusion

we have described is inevitable, and the timescales unreasonably long. The exponentially

large search times arise transparently from the factor $(R/N)^{(d-2)}$ in equation (2). This is

of course a restatement of Levinthal’s paradox (Karplus, 1997), but a helpful one, in that

the two necessary assumptions for the paradox to arise are clearly seen. The first just

gives the large dimensionality of hypersphere, the second the flat diffusive landscape.

Put this way, there are two ways of circumventing the problem. One may drop

the assumption of local forces and allow the protein to “fall” towards the single native

state down a “funnel” created by forces whose range permeate the entire volume. As

we have remarked above, however, candidates for such long range forces do not present

themselves. Without recourse to a continuous funnel-shaped landscape, there is only

one other possibility: all diffusive searches take place in low dimensional subspaces of the

full configurational space.

To see how this works, we suppose at first that the $d \sim O(10^2)$ dimensions of the full

folding space are now arranged sequentially so that diffusive searches in one dimension

at a time allow the protein to find “gateways” into the next subspace (we will see how

this may arise naturally in a physical way below). For simplicity we assume that the

kinetics of each diffusive search is single exponential with characteristic time $\tau$. Since

the diffusion is always maintained in some low-dimensional subspace of the full folding

space, $\tau \sim R^2/D$ for each subspace, so that $\tau_{fold} \simeq d^2 R^2/D$ rather than the exponentially

larger $(R^2/R_N^2)^d$. This clearly reduces the folding time enormously, signifying that only

a tiny fraction of possible states is visited in the search (Dinner et al., 2000).

How has such a remarkable reduction in folding time been achieved without the use

of a “funnel” energy landscape? Of course energetic interactions have been implied, but

these have not been of the spatially-extended “funnel” type. Instead they have served

just to keep the diffusive search within the smaller $(d-1)$ or $(d-2)$ dimensional space,

once the first sub-dimensional search is over, then within a $(d-3)$ or $(d-4)$ dimensional

space after the subsequent successful “adsorption” into the still-smaller subspace, and

so on. So, when the high-dimensionality of the search space is retained, the energy

landscape looks less like a funnel, and more like a series of high-dimensional gutters
The diffusing particle (representing of course the random search of the protein through its available conformations) does not have to search simultaneously through both the dimensions of the figure. Instead, it exploits the lower energy state of the entire \((d - n - 1)^{th}\) dimensional subspace to reach it \textit{via a one-dimensional diffusion in the} \((d - n)^{th}\) dimension, which it performs first. By partitioning the configurational space in this way, and by providing an attractive “gutter”, relying on \textit{local} forces alone, to connect one diffusive subspace to the next, all the advantageous consequences of a funnel landscape may be acquired without the requirement of long-range potentials. Of course, if the high-dimensional structure is projected into a 1 or 2 dimensional diagram then the many discrete steps of potential energy that arise from the sequence of “hypergutters” appear artificially close, and serve to create a funnel-like projected energy landscape. The disadvantage of the projection is that the subtle origin of the directed search is obscured. In detail the folding energy landscape will look more like a series of low-dimensional terraces (inset to figure 1) nested within the full high-dimensional search space.

How big do the attractive potentials creating the gutters need to be, and what physical interactions might be enlisted to provide them? Their scale is familiar: these potential steps are just the energies required to counterbalance the entropy-loss associated with reduction of the configuration space by one dimension, or degree of freedom. The associated translational space reduces from the order of \(R\) to the order of \(R_N\) on restriction to the gutter subspace. Completely reversible folding along the route connecting the gutters is produced by rendering the free energy change on entering the gutter zero. This is in turn the case if the binding energies to the gutters are of the order of the entropic free energy gain on making such a restriction to a degree of freedom:

\[
\Delta U_{gutter} \simeq k_B T \ln \left( \frac{R}{R_N} \right)
\]  

(3)

To quantify \(\Delta U_{gutter}\) therefore needs just an estimate of the order of magnitude of the ratio \(\left( \frac{R}{R_N} \right)\), the dimensionless ratio of the sizes of space enjoyed by a degree of freedom in and out of a restricting gutter. As discussed above, a realistic order or magnitude estimate is \(\left( \frac{R}{R_N} \right) \simeq 10\), giving a value for \(\Delta U_{gutter}\) of the order of a few (2-4) \(k_B T\) (or of order 4-8 kJmol\(^{-1}\)) for realistic proteins. The energy scale of a few \(k_B T\) is highly suggestive: we note that the relatively weak, non-native like interactions between residues are candidates for these gutter-stabilising interactions, and that it is not necessary to invoke the strength of native contacts during the diffusive search. This is good news, since there is no guarantee that significant native interactions will form during at least the early phases of search, if at all, and experimental evidence of strong “co-operativity” is to the contrary (Flanagan et al., 1992). Of course we do not assume that the energy-
entropy balance is exact at each step - indeed it is the mismatches in this picture that give rise to roughness in the landscape, but matching within a few $k_B T$ is necessary in most dimensional reductions to avoid unrealistically long folding times.

Furthermore, the evolutionary tailoring of non-native interactions provides additional “design-space” within which a pathway to the folded state may be coded, but without compromising the stability of the final, native state. For proteins containing $N$ residues, there are of order $N^2$ non-native interactions that may be encountered during a diffusive search, but only of order $N$ interactions that define the native state. A second consequence of this high-dimensional viewpoint is therefore the general expectation of tuned but non-native, interactions between sections of partially structured chain that stabilise intermediate search spaces (which may or may not be identified as intermediate states, depending on their occupancy lifetime). We need to articulate carefully what is meant in this context by “non-native”, for this term is sometimes used to refer to indiscriminate interactions. In that sense, the role of non-native interactions in determining the type and rate of folding pathways is not a new idea (Zhou and Karplus, 1999). But such previous studies have not introduced any specificity, or evolutionary refinement, into the non-native interactions, and find, significantly, that increasing the strength of such indiscriminate interactions actually slows folding. Our suggestion is that a discriminating design of key non-native interactions may significantly speed the search for the native state. It is also likely that a significant proportion of such tailored non-native interactions that we envisage guiding the search will be increasingly near-native as the search proceeds. This will be the more likely as secondary structure forms, as we shall see by the example of a three-helix bundle below.

Gutter-like landscapes have appeared in the literature, and are sometimes apparent even in the 2-dimensional representations of projected folding surfaces. Reference (Karplus and Weaver, 1994), for example, shows a fast folding route of hen lysozyme in which the early formation of $\beta$-sheet structure permits the final approach to the native state to proceed in a subspace of reduced dimension. In this case the gutter-like structure survives a projection onto just two dimensions of folding space. In this case the mutual diffusion of the helical and beta-sheet portions of the protein is the dynamical process responsible for the gutter-like feature on the reduced folding surface. This example serves also to indicate an important qualification - some dimensions clearly do possess funnel-like landscapes even without a projection onto low dimensional spaces. Those involved with the formation of a local $\alpha$-helix or $\beta$-turn structures, for example, create subspaces that have real funnel-like features, directed towards the point in the subspace representing the formation of the complete local secondary structure. However, higher-dimensional hypergutters must already have been visited at higher levels in the regions
of locally $\alpha$ and $\beta$ secondary structure. We now take a much simpler fold as an example.

3 \hspace{1em} \textbf{An example: three-helix proteins}

A clean example of a “hypergutter” structure is furnished by the well-studied triple-helix proteins such as the B-domain of staphylococcal protein A (BdpA) (Myers and Oas, 2001) (and see figure 2). In this case, the division of the folding landscape is clearly suggested by the formation of the helices (fast “funneled” or “zipper” dimensions (Fiebig and Dill, 1993), and by the diffusive search of the helical domains for their native juxtaposition. Note that we do not require the helix formation to be complete before the diffusive search begins - indeed the formation of native or non-specific contacts and secondary structure stability will in general be highly cooperative (Fersht, 2000). All that is required is that the zipper dimensions are explored at much faster timescales than the diffusive dimensions. A very simple model has been successful in describing the kinetics of this protein (Myers and Oas, 2001), using the physical abstraction normal to “diffusion-collision” models of the real protein as spherical domains executing a spatial search. Such models have recently been extended to a family of three-helix bundle proteins (Islam et al., 2002). However, in the light of our expectation that fast-folding proteins find their native state \textit{via} a sequence of stabilised subspaces, the diffusive degrees of freedom of a three-helix bundle might be more accurately represented by angular coordinates defined at the two turns connecting the three helical sections. In fact the diffusive space of internal angles thus defined is exactly three dimensional: between helix 1 and 2 only one angle needs be specified, while between helices 2 and 3 we need two more. This construction is illustrated as in figure 2. The three angular diffusive degrees of freedom are labelled $X_i$ with $i = 1, 2, 3$. Since the diffusive coordinates are angles, they exhibit periodicity, and the search space is itself a periodic 3-d lattice. In practice the continuously varying angular co-ordinate may model a more discrete set of more or less favourable packings (Chothia et al., 1981), but the coarse-grained structure of the search space will be the same. In the figure we illustrate periodicity in the dimension $X_2$ only. The region of configuration space in which the first two helices are both in contact with the third is shaded, and the native state is represented by the periodic lattice of small spheres. If the shaded “helical contact” region is enhanced by a weak attraction (it becomes a “gutter” for diffusion in the $X_2$ coordinate), then the search for the native state will typically proceed by diffusion in the one-dimensional manifold of $X_2$ (without contact between helices), followed by diffusion in the two-dimensional manifold of $X_1$ and $X_3$ (now with helices 1 and 3 in contact). As calculated in the last section, the non-native binding potential of the third helix to the gutter sub manifold needs to be of the order of
3k_BT. Providing that the gutter is as attractive as this, then the predicted mean search time (including prefactors and a weak logarithmic term) for the native state is

$$\tau_{1/2} = \left( \frac{R^2}{D} \right) \left[ \frac{16}{\pi^2} + \frac{1}{2} \left( \ln \frac{R}{R_N} - 1 \right) \right]$$

rather than the much longer time for the full 3-d search without the gutter subspace of

$$\tau_3 = \left( \frac{R^2}{D} \right) \left( \frac{R}{R_N} \right) \left( \frac{4}{3\pi^2} \right)$$

Examples of experimental evidence for staged diffusive searches in simple proteins has also been observed in the case of cytochrome C, lysozyme (Bai, 2000), and in the B1 domain of protein G (Park et al., 1999).

The 3-helix example illustrates our general conclusion that searches within diffusional subspaces in protein folding may be accelerated by local, but not necessarily native, interactions between sections of partially structured chain. In the context of the three-helix protein the necessary non-specific interactions are those that keep the third helix in contact with the other two. This permits the final diffusive search for the native state to take place in a 2-dimensional space, rather than the full 3-dimensional search configuration space of the diffusive degrees of freedom. Remarkably, just this conclusion was reached very recently by experiments on the helical immunity protein Im7 (Capaldi et al., 2001), in which an on-pathway intermediate state was shown by careful mutation studies to be stabilised by non-native interactions between two of the helices. An additional example of tuned non-native interactions guiding a folding pathway occurs in the rather larger Phage 22 Tailspike protein (Robinson and King, 1997), where a non-native disulphide bond controls the folding search. We remark that in both these cases, the stabilised hypergutter provides an arena in which “diffusion-collision” calculations can operate within a molten globule, so constituting a significant generalisation of that model to non-spatial degrees of freedom (Zhou and Karplus, 1999).

## 4 Predictions of the Hypergutter model

We have identified two general predictions of this high-dimensional view of folding: (1) the sequential diffusive exploration of low-dimensional subspaces favoured by fast folding and (2) the stabilisation of these subspaces by discriminate but non-native (or near-native) interactions, without recourse to long-range guiding forces. But it has other things to say concerning common experimental measures of even the deceptively simple “two-state” folders. We derive here three further consequences: (3) early-time structure in kinetics, (4) temperature and denaturant dependence and the free-energy structure of the folding pathway, (5) non-native contributions to Φ-values.
4.1 Relaxation functions in folding kinetics

We first take a very simple case: if the non-native gutter-stabilising interactions are perfectly balanced with the entropy changes at each stage of the dimension reduction, then the free-energy profile is itself flat, and the $d$ diffusive dimensions form an effective one dimensional path along which the folding takes place. This is not, of course, to suggest that the path is unique, since: (i) a large fraction of each sub-dimension may be explored, (ii) the path is at each stage reversible and (iii) the non-diffusive “zipper” dimensions describing the local folding of secondary structure are perpetually exploring their own configurational space rapidly and cooperatively with the slow dimensions. Nonetheless, casting the high-dimensional problem into this form shows that a naïve “reaction co-ordinate” picture can actually emerge from the concatenation of the sequentially-stabilised hypergutters.

An effective 1-dimensional coordinate, $X$ arises from such concatenation of the gutter dimensions of a very high dimensional space, whose initial condition (for a quenching experiment) will favour the high entropy of the early dimensions: every initial state is completely disordered, and the resulting one-dimensional diffusion equation will be supplemented by the approximate initial condition $p(X,0) = 2\delta(x)$. If the native state is representes by a sink for diffusers at $X = 1$, it is straightforward to calculate the fraction of unfolded proteins after a quench as:

$$\sum_{n=0}^{\infty} \frac{4(-1)^n}{\pi(2n+1)} \exp\left(-\frac{(2n+1)^2}{4t}\right)$$

which we plot as a solid line in figure 3. For most of its trajectory, this function mimics a single exponential, but with an effective delay from the moment of quench. This arises from the time it takes for the higher subspaces to be filled - at first the native configuration is “screened” by virtue of being buried in a cloud of states of low entropy. The apparent delay would be noticed only in experiments able to capture the very fastest kinetics after a quench.

4.2 Temperature dependence and the folding pathway

It is very common to represent diagrams of the folding and unfolding rates $k_f$ of proteins plotted against the concentration of denaturant, the so-called “chevron plot”. A more challenging experiment is represented by the Eyring plot ($\ln k_f$ with $1/T$). These plots do not typically show the simple linear form characteristic of chemical reactions with a transition state free energy that is itself independent of temperature. In the case of protein folding reactions they are generally negatively curved, and may contain discontinuities of gradient (Oliveberg et al., 1995). A common interpretation is to claim that
the local gradient of the Eyring plot gives the activation enthalpy at that temperature, and therefore that curvature implies a change in the enthalpy of the transition state. Possible causes suggested have included melting of the differently-sized hydration shells in the unfolded and transition states.

Such a curvature in Eyring plots is, however, a natural consequence of the hyper-gutter model. Low-dimensional diffusive subspaces of progressively lower entropy are stabilised by increasingly negative non-specific binding energies. On average the energies (enthalpies) of the subspaces towards the native state must have large negative values, since the folded native state has such a low entropy compared to the fully denatured state. The enthalpies $\Delta H_n$ of the $n^{th}$ diffusive subspace must attempt to counterbalance their increasingly large (negative) entropies $T \Delta S_n$.

Without any further information on the implicit optimisation of these sets of energies and entropies, but with only one overall constraint that the total free energy to fold $\Delta G_{D\rightarrow N}$ be a fixed value (at some temperature, zero at the folding temperature $T_f$), we can define, as in the last section, a free energy pathway as one-dimensional random walk through the diffusive subspaces (see figure 4). The transition state is the diffusive subspace with the highest free energy. From the figure, it is clear that this corresponds to the greatest positive excursion of the random free energy trajectory. It is also clear that, at the folding temperature $T_f$ (figure 4(b)) this tends to occur midway through the walk, at the point least controlled by the boundary conditions at the endpoints. Well into the folding regime of $T < T_f$, however, this maximum excursion is much more likely to be near the unfolded state, $D$.

Calculations of the mean excursions of a random walk of $Q$ steps of an average energy-difference $\epsilon$ and constrained to a total (negative) free energy change $\Delta G_N$, can be parameterised by the dimensionless quantity $x \equiv \left( \frac{\Delta G_N}{\epsilon} \right) Q^{1/2}$ (see appendix A). In terms of $x$, the expectation value of the transition state free energy is the surprisingly universal form

$$\langle \Delta G^\dagger \rangle = \sqrt{\frac{1}{4} \left( 1 - \sqrt{\frac{x}{1+x}} \right) \left( 1 + \sqrt{\frac{x}{1+x}} \right) - x \left( 1 - \sqrt{\frac{x}{1+x}} \right)}$$

This form may be recast as an Eyring plot using $k_f \sim \exp \left( \frac{\Delta G_N}{\epsilon T_f} \right)$, together with the assumption of a linear dependence on temperature for the depth of the native state energy near the folding temperature itself, so that $x = \beta \left( 1 - \frac{T}{T_f} \right)$, with $\beta$ a dimensionless number. The dimensionless Eyring plot for the parameter $\beta = 1$ is given in figure 5. The curvature of the plot comes from the mean shift of the transition state to more denatured diffusive subspaces along the folding pathway. Alternatively, it can be interpreted as a particular prediction for the Hammond shift of the transition state for high-dimensional protein folding with only the minimal requirements of overall entropy and enthalpy.
balance at each entry down the sequence of gutters. Qualitatively, the effect is close that seen in several cases, and with a comparable magnitude (Oliveberg et al., 1995).

There are, of course, several caveats attached to such a general calculation. Clearly this crudest form cannot pick up specific and discontinuously large shifts of the transition state, which in small proteins will often dominate particular cases (see, for example, the two regimes of temperature for which continuously-curved Eyring plots hold in the case of barnase (Oliveberg et al., 1995)). Nor does it anticipate specifically evolved favourable departures from the random imbalances of entropy and energy assumed here, which certainly arise and roughen the landscape, nor does it account for specific behaviour from hydration shells. Nonetheless, we can see that the qualitative features of temperature-dependent folding do arise without any special assumptions of these kinds. Furthermore, it suggests a rather general structure for the free-energy along a folding pathway, in which successive fluctuations in entropy and energy create a sequence of intermediate states. This type of structure has been investigated theoretically (Wagner and Kiefhaber, 1999) and evidence for its rather general emergence has arisen experimentally very recently (Pappenberger et al., 2000; Sanchez and Kiefhaber, 2003).

4.3 Phi-Values: non-native contributions

As a final general prediction, and as an example of the specific calculations possible with the model, we examine the important question of Φ-value analysis and its interpretation. When the mutation of a residue gives rise to a value of Φ close to 1, it means that the change in folding rate arising from the mutation is consistent with comparable changes in the transition state and native state energies. This is usually interpreted to mean that the residue in question enjoys the majority of its native contacts at the transition state (Fersht, 2000). However, this model suggests another physical source of positive values for Φ, since it identifies non-native interactions as potentially crucial in establishing folding rates. For if a residue contributes via non-native interactions to the stable hypergutter that concludes the dominant (longest) diffusive search, then mutations to that residue will affect the folding rate, even though it does not necessarily possess any native contacts at the transition state. In the hypergutter model, the “transition state” is, by definition, the subspace following that which takes the longest time to search - the rate-determining step.

To make this more precise, we return to the case of the three-helix bundle and calculate the dependence of the total folding rate on the non-native potential that stabilises the 2-dimensional “gutter” of the final search. Defining a “fugacity” $\Delta = \sigma e^{\varepsilon/kT}$, where $\varepsilon$ is the stabilising energy of the 2-d gutter and $\sigma = (R_N/R)$, the measure of the relative sizes of the two spaces, we expect that as $\Delta$ is increased (by increasing $\varepsilon$) we take the
system from the the slower 3-dimensional search to the accelerated “1+2” dimensional search. By adding the currents of diffusers that find the native state from the 2 and 3 dimensional spaces separately, we find an approximate cross-over formula for the folding rate $k_f$ (ignoring weak logarithmic factors):

$$k_f = \left( \frac{\Delta}{1 + \Delta} \right) k_{1+2} + \left( \frac{1}{1 + \Delta} \right) k_3$$  \hspace{1cm} (8)

where $k_{1+2} = \tau_{1+2}^{-1}$ and the slow rate $k_3 = \tau_3^{-1} \approx \sigma k_2$. The expression (8) also contains, by implication, a prediction of the contribution of the non-native interactions to the $\Phi$-values of the residues that contribute to it. For, a mutation of any residue will change its contribution to the gutter potential, so

$$\Phi_g = \frac{1}{n_g} \frac{\partial \ln k}{\partial \varepsilon} \approx \frac{\Delta}{n (1 + \Delta) (\sigma + \Delta)}$$  \hspace{1cm} (9)

where $n_g$ is the number of residues that share the burden of providing the non-native gutter potential $\varepsilon$. We have also assumed in the derivation of (9) that $\varepsilon$ is also the scale of a single residue’s contribution to the stability of the native state - but other reasonable assumptions will only introduce an order-1 prefactor. The functional dependence of $\Phi_g$ on the fugacity $\Delta$ is actually a rather weakly varying function once the gutter is large enough to produce a reasonable fraction of the maximum acceleration of the folding rate, and is close to the value $0.6/n_g$ (it possesses a broad maximum of this value when $\Delta \simeq \sqrt{\sigma}$, see figure 6, which shows how both folding rate and $\Phi_g$ depend on the gutter potential). This non-native contribution to $\Phi$ will naturally be weak in the two limits of vanishing gutter-potential (when all searches are high-dimensional) and very high gutter potential (when they are always low-dimensional).

Again, a rather general result emerges that may be compared with rate measurements on selectively-mutated systems. For the three-helix bundles, contributions to the stabilising potential that encourages the terminal helices to diffuse in contact with each other will arise typically from one residue per helical turn, so that $n_g \lesssim 10$ (by counting about two residues per turn on the contact face of a 5-turn helix). Since the total predicted non-native contribution to $\Phi$ is of order 1 (from the dimensionless function of (9) plotted in figure 6), this means in turn that mutating these residues might generally give non-native contributions to their apparent individual $\Phi$-values of order 0.1. The inset to figure 6 displays the expected pattern of such enhanced $\Phi$-values against residue index. Remarkably, this is precisely what is seen, again in very recent experiments, on the immunity family of helical proteins Im7 and Im9: the member of the family with an on-pathway intermediate (Im7) also exhibits increased $\Phi$-values in the appropriate region of the helices 1, 2 and 4 (helix 3 only forms co-operatively with the native state) by just this amount, relative to the protein without the intermediate, Im9 (Friel et al., 2003).
The magnitude of incremental contributions to $\Phi$ from the gutter potentials is restricted to these low values only in very simple topologies such as the three-helix bundle. When key stabilising interactions succeed in reducing the dimensionality of the search space more drastically, much higher values can result (from differentiation of the higher-dimensional analogues of (8)). In more complex spaces of mutual diffusion of helices and $\beta$-turns, values greater than 1 are not unexpected. This approach suggests a natural interpretation of $\Phi$-values greater than one, such as recorded in acylphosphatase (Chiti et al., 1999), but which do not bear an interpretation in terms of native structure (Fersht, 1999).

The interpretation of non-classical $\Phi$-values outlined here is closely-related to a recent suggestion arising from some simple lattice Go-type simulations (Ozkan et al., 2001). The simulation also found that kinetic properties are more closely connected with $\Phi$ than local “degree of nativeness”. It shares with the present treatment the essential departure from a one-dimensional projection of a transition state, and an identification of the number of permissable pathways, or transition entropy, in controlling the rate of folding.

5 Relationship to other models

The model of high-dimensional diffusive hypergutters is not incompatible with the frameworks or results of the other models discussed in the introduction, but rather serves to show how the apparently alternative models are related. Each emerges from the high-dimensional hypergutter picture when a different projection into a low-dimensional space is applied.

When the flat, diffusional, freedom are projected away onto a reaction co-ordinate pair such as $R_g$ and $\phi_{\text{native}}$, then a folding funnel appears, and does so without the presence of any long-range forces. The difference is that, on close examination, the funnel is discrete, or terraced, rather than continuous. Furthermore, it appears when the interactions generating coil-collapse are projected along an ordinate of sequential sub-spaces, rather than along a spatial co-ordinate. But when there are many sequential subspaces, an apparently continuous folding funnel appears with all of the features of intermediate states, multiple pathways etc. ascribed to it (Brygelson et al., 1995; Dinner et al., 2000) arising in a natural way. Another example of this projection is found in the master-equation approach (Zwanzig, 1995), in which the smoothly funneled high dimensional energy landscape implies some shaping of the non-native contacts of the underlying model.

When the projection is orthogonal to one of the later diffusional subspaces, on the other hand, then the same system will appear to map onto a diffusion-collision model.
In this case the projection concentrates on the diffusive degrees of freedom one by one, rather than projecting them away into a funnel. The advantage of the hypergutter approach, however, is that it identifies diffusive subspaces in cases where the standard diffusion-collision model does not. The case of diffusion in mutual angular space of helical bundles discussed above is an example, since this occurs within a globule, rather than in the collisional formation of a globule. It also recognises intermediate cases in which diffusional searches occur simultaneously in high and low-dimensional spaces, such as a partially-stabilised 2-d gutter in the three-helix case, and provides a structure for introducing tailored, rather than indiscriminate, non-native interactions. The interesting and unexpected prediction of non-native and positive $\Phi$-values emerges in just this case.

Of particular interest is the relationship of the hypergutter picture to the topomer search model. This is because the rate-determining diffusive searches will in general be completed only when a topological, as well as a spatial, constraint in the final native state is satisfied for the first time. It is also to be expected since this model also seeks to understand folding rates without recourse to their dependence on native interactions. Again, the three helix bundle serves as a model example—restriction from the 3-d helical angular space to the faster 2-d space with helices 1 and 3 in contact occurs when the topological orientation of the helices is satisfied. Similar conclusions would emerge if the slow-searches were between a helix and a $\beta$-sheet, or between two or more $\beta$-turns. Retaining all the diffusional degrees of freedom leads to a close relationship between the contact order $Q_D$ of the topomer search model, and the number of diffusional dimensions $d$ of the space of hypergutters in the rate-determining diffusive search. For we can identify the exponent $Q_D$ in the rate expression in the topomer search model (Makarov and Plaxco, 2003)

$$k_f \sim \gamma \langle K \rangle^{Q_D}$$  \hspace{1cm} (10)

with that in the diffusive-search result (2) above to find, formally at the level of the exponent that $Q_D = (d - 2)$ where here $d$ is the dimension of the largest diffusive search. We note, however, that departures from the correlation of folding time with contact order might be expected when non-native interactions are tuned to speed up folding in the way we have outlined. This is because such a strategy can reduce the effective dimension of the search without changing the topology of the final state. Strong outlying behaviour in the correlation of folding time with contact order may be connected with the potential variability in the efficiency of hypergutter stability portrayed in figure 6.
6 Discussion and Conclusions

We have discussed a conceptual approach to the protein landscape problem that attempts to remain faithful to the high dimensionality of the system. Rather than invoking a continuous energy landscape with long-range forces giving rise to a funneled landscape, we use rather general considerations to point to a high dimensional structure of “hypergutters”. These structures describe the search for the native state as a sequence of relatively low-dimensional diffusive subspaces. Only spatially-local interactions are required to direct the folding towards the native state in reasonable times. As a by-product, this procedure also draws together into a single picture the apparently divergent views of the folding funnel, collision-diffusion, nucleation-condensation and topomer search models. The rate-determining “gutter” dimensions lie orthogonal to other “zipper” dimensions describing the local formation of secondary structure, that are characterised by a continuous folding funnel.

Looked at another way, our structure is a more detailed examination of the sort of dynamic processes that must be occurring within the “molten globule” phase of protein folding. The formation of the globule itself from the denatured state corresponds in this picture to the first hypergutter in a series. It is clearer in experiment than the subsequent dimensional reductions because it is the only one that makes significant changes to the radius of gyration of the protein.

We might note that such a pattern of non-specific binding in diffusive searches is a common motif in biology, appearing for example in the search of DNA-binding repressors for their operons (Winter et al., 1981). In this case, the slow search for a specific binding site in \( d = 3 \) is substituted for a much more rapid diffusive search in \( d = 1 \) (along the DNA) by non-specific binding of the repressor proteins. In this process too, there is strong evidence that the non-specific interactions are themselves subtly coded to further speed the search for the binding target.

Several general predictions follow. The first is that special tuning of non-native interactions may contribute significantly to rapid folding; they stabilise the hypergutter-potentials that keep diffusive search dimensions under control. In the case of helical proteins, candidates for the structure of the gutters are non-specific contacts of the helices, and the angular, rather than translational, degrees of freedom describing their mutual configurations. In proteins with more complex structures, other candidates suggest themselves, such as the orientation of helices with respect to \( \beta \)-sheets with which they are in contact in \( \alpha/\beta \) proteins, and the relative orientations of \( \beta \)-turns and partially-folded \( \beta \)-sheets in all-\( \beta \) elements. Very recently, the role of non-native interactions in stabilising an on-pathway intermediate, together with a diffusion-collision kinetic route, has been experimentally verified in the case of the immunity protein Im7 (Capaldi et al.,
This precisely exemplifies the general mechanism we have suggested, with the additional feature that one of the hypergutters has become so stabilised (and therefore so populated) that it attracts the label of “intermediate state”. Other examples of non-natively stabilised folding pathways are emerging (Robinson and King 1997). Perhaps the most remarkable example is the determination by fast kinetic experiments that β-lactoglobulin employs an transient helical motif that is entirely non-native (Park et al., 1999). By stabilising a β-turn that otherwise relies on highly non-local, and late-forming, structure for its stability, this temporary helix reduces the dimension of the search space for the non-local contacts. Of course, it is not impossible to achieve the dimensional reduction we have outlined by using fully native, rather than non-native interactions. Such proteins would present a highly bimodal distribution of Φ-values, clustering closely to 0 and 1. A candidate would be acylphosphatase (Paci et al., 2002) in which the “transition state” strongly constraints the environment of just three residues and the immediate neighbours. In the hypergutter picture, the nine-dimensional search space of these critical regions separately is reduced to three sequential three-dimensional searches by the long lifetimes of the native regions when the remainder of the protein is disordered.

One possible advantage of using broadly distributed non-native interactions, rather than a few local native ones, to stabilise hypergutters, is that the intermediate states are thereby more tightly confined. This is turn may assist in suppressing the pathway to aggregated states, or amyloid formation (Dobson, 2002). This suggestion has recently been made from observations of competing folding pathways in a β-sandwich protein.

As both the sensitivity and time-resolution of kinetic experiments increases, finer details of the intermediate diffusional subspaces in this and other proteins should become equally transparent. Another recent, theoretical, contribution has pointed out that fine-structure of a few $k_B T$ within the transition state on a reaction pathway can accelerate folding (Wagner and Kiefhaber, 1999). As an example of the type of fine-structure predicted, the model contains a natural explanation of the generic curvature seen in Eyring plots of the temperature dependence of folding rates. The pseudo-random differences in the enthalpy and entropy of the transitions from one diffusive subspace to the next result in the transition state energy becoming typically more and more negative as the quench depth increases. This Hammond-like behaviour will contribute at least in part to the experimental signals, and suggest the gathering of a wider data sets of this type. Very recent findings suggest that a structure very similar to this predicted one is indeed rather general (Sanchez and Kiefhaber, 2003).

Features of the time-dependent folding curves as functions of temperature or denaturant also follow from the model, including the possibility of an apparent delay before single exponential kinetics set in. It is also possible that “kinetic traps” arise not just
from low-energy intermediate states, but from intermediate diffusive subspaces of higher dimension than 2, for which the control of dimensionality has been incomplete. This is significant for the topomer search model: we might expect to find departures from the folding time/contact order correlation when, in spite of sharing the same topology of fold, one protein in a pair has an important diffusional subspace stabilised while the other does not. Alternatively, our picture suggests ways of increasing folding times greatly by selective mutations that retain topology and stability of the native state, but destabilise one or more of the on-pathway diffusional subspaces so that intermediate searches are required in $d > 3$.

The model provides an alternative interpretation of the results of protein engineering analysis, and suggests that not all contributions to measured $\Phi$-values at the transition state arise from native-like interactions. It suggests interpretations for $\Phi$-values of order 0.1-0.2, but also indicates that contributions to larger values (including the non-classical range $\Phi > 1$) may arise from non-native interactions with that residue that serve to restrict the folding space. More detailed predictions of non-native contributions to $\Phi$ for the family of bacterial immunity proteins and their mutants are in accord with very recent experiments.

Finally it suggests that the “kinetic code” that informs the search for the native state may be found in evolved selection of some of the non-native interactions. The large number of these, of order $N$ times greater than the number of native interactions, make them a likely candidate for information storage, as well as their natural propensity for kinetic control.

The framework and specific examples discussed here suggest useful coarse-grained models of other families of proteins that may be simulated very efficiently, or even approached analytically, as we have done with the 3-helix bundles. Strong experimental evidence is currently emerging that supports all of the main predictions of the approach; other experimental tests of the more surprising conclusions are awaited.

A Diffusive subspaces and random energy excursions

We consider the free-energy as a directed one-dimensional walk $W$ in energy space of $Q$ steps each of mean (free) energy $\epsilon$. We first consider the case of return to the origin, which corresponds to the folding temperature $T_f$ at which $\Delta G_{D \rightarrow N} = 0$. To find the typical excursion of the walk we may map the problem onto a one-dimensional polymer (so that the free-energy walk itself has a free energy $F(W)$). At a coordinate $z = n/Q$, corresponding to the $n^{th}$ diffusional subspace of the $Q$ total, this free energy of the walk is composed of the segment before $x$ and the segment after. If $\Delta G(z)$ is the free energy at
coordinate \( z \), the free energy of the whole walk, integrating over all other possible energies for the individual subspaces is the sum of the two “entropic elastic” contributions from these pieces:

\[
F(W) = \frac{(\Delta G(z))^2}{\epsilon^2 Q z} + \frac{(\Delta G(z))^2}{\epsilon^2 Q (1 - z)} \tag{11}
\]

Since the probability for energy excursions \( \Delta G(x) \) is proportional to \( e^{-F(W)/k_B T} \), the distribution of \( \Delta G(x) \) at each point is Gaussian, with mean

\[
\langle \Delta G_{\text{max}}(z) \rangle = \epsilon Q^{1/2} (z - z^2)^{1/2} \tag{12}
\]

which peaks, of course, at \( x = 1/2 \).

Now taking the directed random walk, the expected maximum value of the free energy at \( x \) is just the random excursion calculated above superimposed on a steady drift towards the native state \( x = 1 \) at the native free energy. Taking out constants with dimensions we write:

\[
\frac{\langle \Delta G_{\text{max}}(z) \rangle}{\epsilon Q^{1/2}} = (z - z^2)^{1/2} - xz \tag{13}
\]

where the parameter \( x = |\Delta G_{D-N}| / \epsilon Q^{1/2} \) is a measure of the quench depth. It is a simple matter to maximise this function over \( z \), and then to find the maximum value. The result is equation 7.

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Figure Legend

Figure 1
Part of the $d$-dimensional folding space containing a diffusive hypergutter projected onto 2 dimensions. The diffusing particle (representing the random search of the protein through its available conformations) does not have to search simultaneously through both the dimensions of the figure. Instead, it exploits the lower energy state of the entire diffusive subspace of the $(d - n - 1)^{th}$ subspace to reach it via a one-dimensional diffusion in the $(d - n)^{th}$ dimension.

Figure 2
The 3-helix bundle (BdpA on the left) is coarse-grained to a system of three rods. The three angles constituting the diffusive subspaces are labelled $X_i$ for $i = 1, 2, 3$. The folding space then looks like the periodic cubic lattice on the right (only the $X_2$ direction is shown periodic, for clarity). The attractive gutter is the 2-d space spanned by $X_1$ and $X_3$ once $X_2$-diffusion has brought the third helix into contact with the other two. But for small angles $X_2$ there is a large topological barrier between the “correct” and “incorrect” sides of attachment of the third helix onto the bundle formed by the other two, and identical with the rapid diffusional subspace of $X_1$ and $X_3$.

Figure 3
Log-linear plot of three relaxation functions. Dashed is the single exponential. Dotted is the decay to the native state for 1-dimensional diffusion with a uniform distribution of initial states. Solid curve is the decay of an effective 1-dimensional folding path created from a high dimensional landscape with flat free energy.

Figure 4
The one-dimensional folding free-energy in terms of sequential diffusive subspaces. (a) $T < T_f$; the landscape is a directed random walk - the maximum excursion (transition state) lies towards the start of the walk. (b) $T = T_f$; the walk returns to the origin of free energy - the maximum lies near the middle of the trajectory.

Figure 5
Dimensionless Eyring plot of the universal form in the version of the gutter-landscape model in which only the energies of native and denatured states are specified (to convert to numbers on an experimental Arrhenius plot, the figures on the ordinate should be multiplied by the square root of the number of diffusive dimensions, or contact order, of the protein). The inset shows experimental results on the protein CI2, from [?].

Figure 6
Predictions of the folding rate (solid line) relative to the rate of the optimal 1+2 dimensional search path, and sum of non-natively generated φ-values from residues contributing to a 2-d diffusive hypergutter (3 helix bundle) (dashed line). The ordinate is the “fugacity” measure of the attractive potential \( \Delta = \sigma e^{\varepsilon kT} \). The value assumed for the spatial reduction \( \sigma \), is 0.1. The inset contains the expected magnitude of increase in \( \Phi \)-values with residue index (dashed lines) in a 3-helix protein with a 2-d kinetic intermediate gutter (Im7), relative to one without (Im9). We expect the modifications to be concentrated onto helices 1 and 4, whose mutual contacts stabilise the 2D search space.
Figure 1: Schematic of sequential searches in gutters
Figure 2: Coarse-graining of the 3-helix bundle
Figure 3: Delay-correction to single-exponential kinetics
Figure 4: Random walk of gutter energy landscape
Figure 5: Predicted curvature of Eyering plot
Figure 6: Dependence of folding rate and effective phi-value on gutter potential