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FINDING THE LOWEST FREE ENERGY CONFORMATION OF A PROTEIN IS AN NP-HARD PROBLEM: PROOF AND IMPLICATIONS

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The protein folding problem and the notion of NP-completeness and NP-hardness are discussed. A lattice model is suggested to capture the essence of protein folding. For this model we present a proof that finding the lowest free energy conformation belongs to the class of NP-hard problems. The implications of the proof are discussed and we suggest that the natural folding process cannot be considered as a search for the global free energy minimum. However, we suggest an explanation as to why, for many proteins, the native functional conformation may *coincide* with the lowest free energy conformation.

Introduction.

Protein folding. The protein folding problem is one of the most important open problems in biochemistry. It can be stated simply: given the amino acid sequence of a protein, calculate the three dimensional structure of that protein. The two experimental methods of determining protein structure, X-ray crystallography and Nuclear Magnetic Resonance (NMR), are still very time consuming while sequencing has become routine and efficient. In the future the large gap between the number of known structures (hundreds) and the number of known sequences (tens of thousands) is likely to explode. Thus, the development of methods to calculate structure from sequence is more important than ever. That the computational problem is difficult is evident from the fact that in spite of extensive efforts during many years, no structure has been reproduced by calculation alone. While many developments have been reported, especially in the field of homologous modeling where new structures are predicted from the known structures of related proteins (see reviews by Blundell *et al.*, 1987; Moult, 1989), the general problem is still beyond our capabilities.

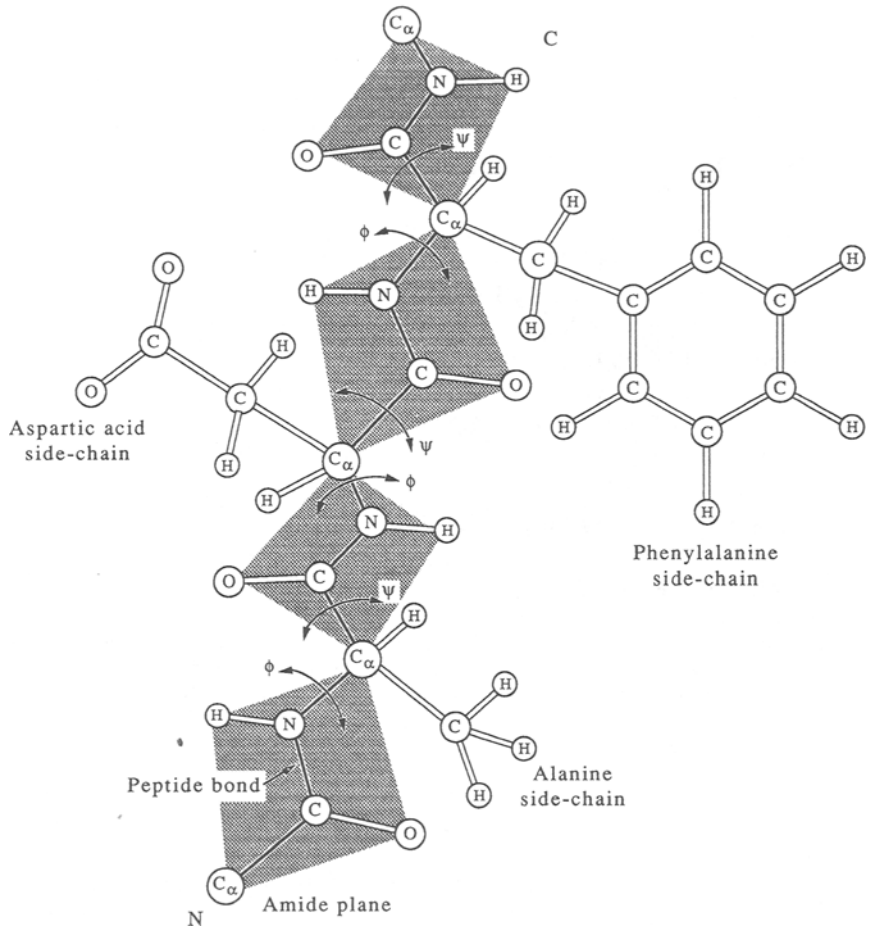

Figure 1. A schematic view of a fragment of a protein chain. The chain runs from the N-terminal to the C-terminal and in this example includes three amino acids. Each amino acid consists of a backbone part and a side-chain. The backbone is essentially the same for all amino acids and consists of planar groups (forming the amide planes which are shaded in the figure) linked by C_α atoms. There are 20 different side-chain types which may be attached to the C_α atoms. The sequence of these side-chains along the backbone determines the three-dimensional structure of the protein, which can be described by the values of the ϕ and ψ angles on either side of the C_α atoms.

A protein is a chain of amino acids linked by peptide bonds (see Fig. 1). Each one of the 20 different amino acids consists of a common main chain part, containing the atoms N, C, O, C_α and two hydrogen atoms, and one of the 20 different side-chains. The side-chain branches out of the backbone at the C_α atom. The two dihedral angles ϕ and ψ on either side of the C_α atom are the

main degrees of freedom permitting the polypeptide chain to adopt different three-dimensional conformations. Actual values of these angles found in proteins are generally restricted to a few distinct domains in the ϕ–ψ space (Ramakrishnan and Ramachandran, 1965; Herzberg and Moult, 1991). The side-chains have additional degrees of freedom, allowing local fine tuning of the structure.

Proteins are capable of folding into their essentially unique native structure without any additional genetic mechanisms. That is, the linear polypeptide chain contains all the information required for the folding process (Anfinsen *et al.*, 1961). This observation is true at least for many small to medium size, soluble, single-chain globular proteins. Recently, a special class of proteins called chaparons have been shown to be involved in folding proteins *in vivo* in the cell environment (see review by Gething and Sambrook, 1992). Their role is still unclear, but it seems to be related to facilitating folding and transport in the complex milieu of the cell. Still, the fact that the sequence contains all the necessary information for folding has been demonstrated repeatedly by the ability to refold proteins *in vitro* without the need of any special folding mechanisms.

As of now there is no accepted theory that explains how proteins fold to their unique native conformations. There are further arguments as to whether the native structure of a protein has the lowest free energy among all the possible conformations of the chain. In this paper we suggest that the native structure cannot be guaranteed to have the global free energy minimum. It is clear that searching for the global minimum in the entire exponential space by enumeration (i.e. scanning through all of the possible conformations) is not feasible. Even if we assume only very few allowed values for the ϕ and ψ dihedral angles, and using the most generous approximation as to how fast these angles can be rotated, it still would take 10^{27} years(!) to explore the whole conformational space for a single, 100 residues long protein (Levinthal, 1969). Generally, having a space that is too large to be searched by enumeration does not exclude the possibility of designing an efficient search algorithm. Here, we will show that the protein folding problem belongs to the class of NP-hard problems and thus no efficient mechanism can be devised to guarantee finding the global minimum.

NP-completeness and NP-hardness. The notion of NP-completeness was invented to describe a class of problems that are "hard" to solve. For all of the problems in the class there exists an *exponential* time algorithm, but a *polynomial* time algorithm is not available for any of them. Consider for example the Hamiltonian path problem: Given a graph (e.g. a road map between n cities), is there a path that visits each node (e.g. city) exactly once? It is clear that by examining all of the exponentially many possible paths one can

decide whether a Hamiltonian path exists, but a polynomial time algorithm is not known. The terms exponential and polynomial measure the type of dependence of the running time of the algorithm (until a solution is found) on the size (based on a reasonable representation) of the data. The dependence is based on "worst case" analysis, namely the time that guarantees to bound the performance of the algorithm on every possible instance of the data. An algorithm is said to be polynomial if the running time can be bounded by a polynomial function in the size n of the problem, and exponential if the dependence is described by an exponential function of n. The distinction between exponential time and polynomial time solutions is crucial because of a very simple fact: exponential functions grow much faster than polynomial ones. While an algorithm that has a polynomial running time (even if the polynomial function is of a relatively high order) is feasible on modern computers even for big problems, exponential algorithms are useful only for very small "toy models". For example, if the size of the problem, n, is 100 an n^5 polynomial algorithm will take 10^{10} time units, which is feasible, but a 2^n exponential algorithm will require about 10^{30} time units, which is prohibitively long. If, for example, the time unit is a microsecond then the polynomial algorithm will take less than 3 hr while the exponential algorithm will require about 10^{16} years(!). For a good introduction to the subject of NP-completeness and NP-hardness see the book by Garey and Johnson (1979).

A problem is said to be *in* NP if we can show that it has a **Nondeterministic Polynomial** solution. In simpler terms this means that when a solution is given it can be varified in polynomial time. For example, in the Hamiltonian path problem when a path is suggested one can in polynomial time (actually in linear time) verify that the path visits each city exactly once. By the above definition every polynomial problem is in NP. To separate the difficult problems among those in the NP class we have the notion of the class of NP-complete problems. The problems in this class are (1) *in* NP and (2) they are the "hardest" in the NP class.

To prove that a problem is NP-complete one has to show first that the problem is *in* NP, and second that some known NP-complete problem can be polynomially transformed to it. The logic behind the second requirement is that if the known NP-complete problem can be polynomially transformed to the new problem then the new problem must be at least as hard. If this was not the case and the new problem could be polynomially solved one could design the following polynomial procedure to solve the known NP-complete problem: Start with an instance of the known NP-complete problem, transform it, polynomially, to the new problem. Now solve the new problem in polynomial time. Since the composition of polynomial functions is a polynomial function, the whole procedure takes polynomial time. As this is impossible it follows that the new problem must be as hard as the known NP-complete problem. The first

problem that was shown to be NP-complete, the Satisfiability problem (Cook, 1971), was of course proven by another method.

The class of NP-hard problem contains the problems that are at least as hard as any NP-complete problem (namely they satisfy the second condition mentioned above), but are not shown to be *in* NP (the first condition above). Thus, the class of NP-hard problems is more general and contains the class of NP-complete problems. One source of difficulty in proving that a problem is *in* NP comes from limitations on the precision of some arithmetic calculations on a finite computing machine.

Another source of difficulty arises from the fact that formally the NP-complete problems are "decision" problems, like the Hamiltonian path problem: "Given a graph, does it contain a Hamiltonian path?", for which the answer is either yes or no. Optimization problems, like the famous traveling salesman problem: "Given a road map between n cities, which is the shortest tour that will visit all of them?", are not directly considered within the NP-complete framework. Actually one can transform an optimization problem to its decision version. In the traveling salesman example the optimization problem can be transformed into a decision problem by introducing a parameter B and asking whether there is a tour shorter than B. This decision problem has been shown to be NP-complete. As the solution of the underlying optimization problem is at least as hard it has been shown accordingly to be an NP-hard problem.

While there is still no proof that a polynomial solution is impossible for the NP-complete problems (the famous NP = P problem), these are generally considered to be a set of problems for which an efficient algorithm cannot be found. We adopt this assumption and regard NP-complete and, of course, NP-hard problems as intractable.

The long and frustrating experience with the protein folding problem indicates that we are dealing with a hard problem. In this paper we will show *formally* that the corresponding computational problem of finding the global free energy minimum is hard. We will prove that a reasonable discrete model of the problem belongs to the family of NP-hard problems. As will be elaborated in the discussion, the implication of the proof is that there is no way to guarantee that the native structure will always be the lowest free energy structure.

The Model. It has long been known that the number of possible foldings of an n-long chain into a lattice (the number of self-avoiding walks on a lattice) is exponential in the length of the chain. This number is given by the following formula: $A\mu^n n^\alpha$, where A is a constant independent of n, α is dependent on the dimensionality and $\mu > 1$ is dependent on the type of the lattice (see review in Mazur, 1969).

In our model we consider a cubic three-dimensional lattice. Each amino acid in the chain is represented by one element which can occupy one cubic cell of the lattice. The model is self avoiding and thus only one element is allowed into each cell. The sequence is folded on the lattice such that two contiguous amino acids in the sequence must reside in two neighboring cells of the lattice. Cells are defined as neighbors if they have at least one lattice point in common. Thus, each amino acid can have 26 possible neighbors. The growing chain can choose any unoccupied cell out of these for the next amino acid (at least one must be occupied by the previous amino acid); see Fig. 2.

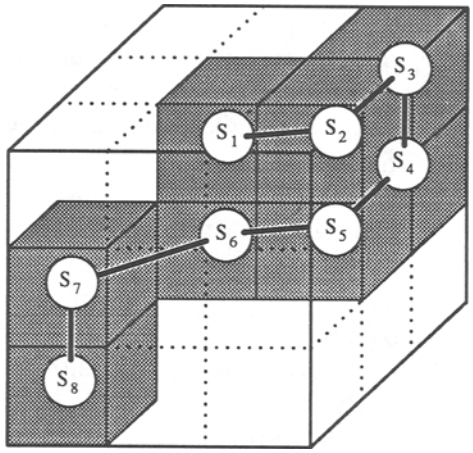

Figure 2. Lattice model of a folded protein. The sequence (in this example $(s_1, s_2, \ldots, s_8)$) is folded into a cubic lattice. Each sequence element occupies one lattice cell. As the model is self avoiding, only one element can reside in each cell. Two contiguous elements in the sequence must be in neighboring cells. Cells are considered neighbors if they share at least one lattice point.

We would like to find the global minimum of some pairwise free energy function, which is dependent on the types of the amino acids in each pair and on the distance between the amino acids. Formally, the protein folding (PF) problem can be stated as: Given a sequence $S=(s_1, s_2, \ldots, s_n)$ we would like to find a one-to-one "folding" function, $\mathbf{f}(s_i)\to I^3$, that given the integer costs of interaction between sequence pairs $c_{s_i s_j}$ and a positive function $\mathbf{g}$: $I^3\to R^+$ of the three-dimensional distance components between the folded elements: $\mathbf{g}(\Delta_x(\vec{l}, \vec{k}), \Delta_y(\vec{l}, \vec{k}), \Delta_z(\vec{l}, \vec{k}))$, where $\Delta_x(\vec{l}, \vec{k})=|l_x-k_x|$, $\Delta_y(\vec{l}, \vec{k})=|l_y-k_y|$, $\Delta_z(\vec{l}, \vec{k})=|l_z-k_z|$ and $\vec{l}, \vec{k}\in I^3$ are lattice coordinates, would minimize the function:

$$E=\sum_{i=1}^{n}\sum_{j\neq i} c_{s_i s_j}\mathbf{g}(\Delta_x(\mathbf{f}(s_i), \mathbf{f}(s_j)), \Delta_y(\mathbf{f}(s_i), \mathbf{f}(s_j)), \Delta_z(\mathbf{f}(s_i), \mathbf{f}(s_j))),$$

such that $\Delta_x(\mathbf{f}(s_i), \mathbf{f}(s_{i+1}))$, $\Delta_y(\mathbf{f}(s_i), \mathbf{f}(s_{i+1}))$, $\Delta_z(\mathbf{f}(s_i), \mathbf{f}(s_{i+1}))\leqslant 1$,

$i=1, 2, \ldots, n-1$ (this distance constraint forces contiguous elements to occupy neighboring cells).

The corresponding decision protein folding (DPF) version introduces a parameter B and asks whether there exists a folding $\mathbf{f}$ such that $E \leqslant B$.

This model captures the essence of the important components of the real protein folding problem. The discretization of possible directions of the chain at each point is justified by the fact that the real ϕ and ψ angles are indeed constrained to specific domains. The modeling of each amino acid by only one point in the model is common (Levitt and Warshel, 1975) and it can be justified because it has been shown that from the set of C_α coordinates (one atom per amino acid) one can generally build a reasonable model of the full protein (for example, see Holm and Sander, 1991). The form of the free energy function that we consider here is very simple, but it can serve as a good first approximation to many of the real terms. The internal energy of proteins in terms of a full atomic description of their conformation (for example, see Brooks *et al.*, 1988) can be approximated with functions of that form. More relevant to our model are the empirical mean force functions, which are used to describe the free energy of lattice conformations and have the same form that we are discussing. These are based on a sum over all residue pairs as a function of residue types and distance. The free energy contribution of each pair is derived from analysis of distances between amino acid types in known protein structures. Such empirical mean force models have been shown to have low values for conformations close to the correct structures (Covell and Jernigan, 1990; Seetharamulu and Crippen, 1991) and thus it is established that they represent the relative free energy of conformations appropriately. As the empirical mean force is derived directly from the coordinates of known structures it must reflect all the free energy components involved in protein folding, including van der Waals interactions, electrostatic forces, solvation energies, hydrophobic effects and other entropic contributions. Still, it is clear that our model is simplified. However, if we can show that this simple model is hard, solving more realistic models would be even harder.

As mentioned above, to prove that the protein folding problem is NP-hard we have to show that some known NP-complete problem can be polynomially transformed to it. As a basis for the proof we use the "optimal linear arrangement" (OLA) NP-complete problem. We will show the polynomial transformation between the problems. Thus, if the decision version of the protein folding problem (DPF) could be solved polynomially then the "optimal linear arrangement" could also be solved in polynomial time, in contradiction to its known status as an NP-complete problem. This will establish the NP-hardness of the DPF problem. Since solving the optimization PF problem would directly suggest an answer to the decision version it will follow that the optimization problem is NP-hard.

The "optimal linear arrangement" (OLA) problem (Garey *et al.*, 1976) is stated as follows: Given a sequence $S'=(s_1, s_2, \ldots, s_n)$, non-negative integer costs $c'_{s_i s_j}$, $1 \leqslant i, j \leqslant n$ and a positive integer B', is there a one-to-one function $\mathbf{f}'$: $(s_1, s_2, \ldots, n) \rightarrow \{1, 2, \ldots, n\}$ such that:

$$E' = \sum_{i=1}^{n} \sum_{j \neq i} c'_{s_i s_j} \left| \mathbf{f}'(s_i) - \mathbf{f}'(s_j) \right| \leqslant B'?$$

This problem can be seen as a one-dimensional mapping assignment, where every element in the sequence $(s_1, s_2, \ldots, s_n)$ should be mapped to a different cell on an n-long one-dimensional lattice. The function that we want to minimize is a pairwise summation of the costs of interactions between the elements times the distance between them. The difference between this problem and the protein folding problem is the connectivity requirement of the latter (i.e. that contiguous sequence elements must be mapped to neighboring lattice cells).

The OLA is a one-dimensional problem. We will take advantage of the two additional dimensions in the DPF problem to transform any instance of an OLA to a corresponding DPF instance, as follows: We will "pad" each element in the original OLA sequence $S'=(s_1, s_2, \ldots, s_n)$ by $3n+2x$s (where x is a special element not in S') to obtain:

$$S = \underbrace{xxx \ldots xx}_{3n+2} s_1 \underbrace{xx \ldots xxx}_{3n+2} s_2 \underbrace{xx \ldots xxx}_{3n+2} \ldots \underbrace{xx \ldots xxx}_{3n+2} s_n.$$

The costs will be:

$$c_{s_i s_j} = \begin{cases} c'_{s_i s_j} & \text{if } s_i, s_j \in S'; \\ 0 & \text{otherwise.} \end{cases}$$

We will use the parameter $B=B'$ to bound the function E.

The pairwise dependence on the three-dimensional distance will be:

$$\mathbf{g}(\Delta_x(\vec{l}, \vec{k}), \Delta_y(\vec{l}, \vec{k}), \Delta_z(\vec{l}, \vec{k})) = \begin{cases} \left|\Delta_x(\vec{l}, \vec{k})\right| & \text{if } \Delta_y(\vec{l}, \vec{k}), \Delta_z(\vec{l}, \vec{k}) = 0; \\ \dfrac{B+1}{C} & \text{otherwise,} \end{cases}$$

where C is the smallest, nonzero cost among the $c_{s_i s_j}$.

An original OLA solution can be extracted from a DPF solution simply by omitting all the xs and leaving all the elements of the original sequence S'.

The combination of the above transformations will guarantee that an accepted solution (i.e. conformation for which the energy is below the limit B) of the DPF is actually an accepted solution for the original OLA problem: The costs were chosen in such a way that only the original elements of S' can effect

the energy and the additional xs will not contribute to the sum. The dependence on the distance was constructed in such a way that elements of S' that are mapped to lattice cells other than those of the original one-dimensional stripe will make the energy too high. When a solution maps even a single element out of the original stripe its energy will be at least $B+1$ (note that because $\mathbf{g}$ is a positive function and the c' of the OLA are non-negative numbers, the energy will never decrease) and the solution will be rejected.

We have to show that the answer to the constructed DPF problem is yes exactly when the answer to OLA is yes. To see that we will show the following two points: (1) That every possible conformation for the original OLA problem has a corresponding (same energy level) conformation for the DPF problem. (2) That an accepted solution of the DPF problem corresponds to an accepted solution of the OLA problem. To see the first point we show that given any mapping $\mathbf{f}'$, the $3n+2x$s between each contiguous pair of original elements are sufficient to connect them. As can be seen in Fig. 3 the original elements are connected, alternately below and above the plane of the original stripe. These connecting chain segments can be built in a way that will ensure that they will not collide with each other, as follows: Each connecting chain will be assigned to a different horizontal plane, so we use $n-1$ additional planes. Elements s_i, s_{i+1} will be connected on plane + (for odd i) or − (for even i) i in the following way: The chain will go down from s_i (alternately, go up) vertically to the horizontal plane i, then leave the vertical plane through the original stripe, complete the turn on its horizontal plane, come back to the point below s_{i+1} and then go up to the original point s_{i+1}. The chains avoid each other because all the vertical movements are done in the vertical plane containing the original stripe and each chain segment is using different columns in this plane going vertically up or down through the original elements. The horizontal movements for the different connections are done in different horizontal planes, avoiding the vertical plane in which the vertical movements are made. The length of the stretch of xs, $3n+2$, is long enough to connect any two elements in this way, as in the worst case we would need to go up and down to the nth plane and then connect elements that are at most n units apart. The additional 2 xs guarantee that movement out of the vertical plane is always possible. In most cases the chain will be longer than necessary, but we can always accommodate the extra chain in its designated horizontal plane without a risk of collision, simply by increasing the distance traveled from the vertical plane containing the original stripe. Diagonal movements can be used to connect original elements regardless of whether the distance between them is odd or even.

In this construction all of the original elements lie on the original stripe and thus make the same pairwise contribution to E as in the OLA problem. All of the xs do not contribute anything as their c is zero. Thus, the energy of this

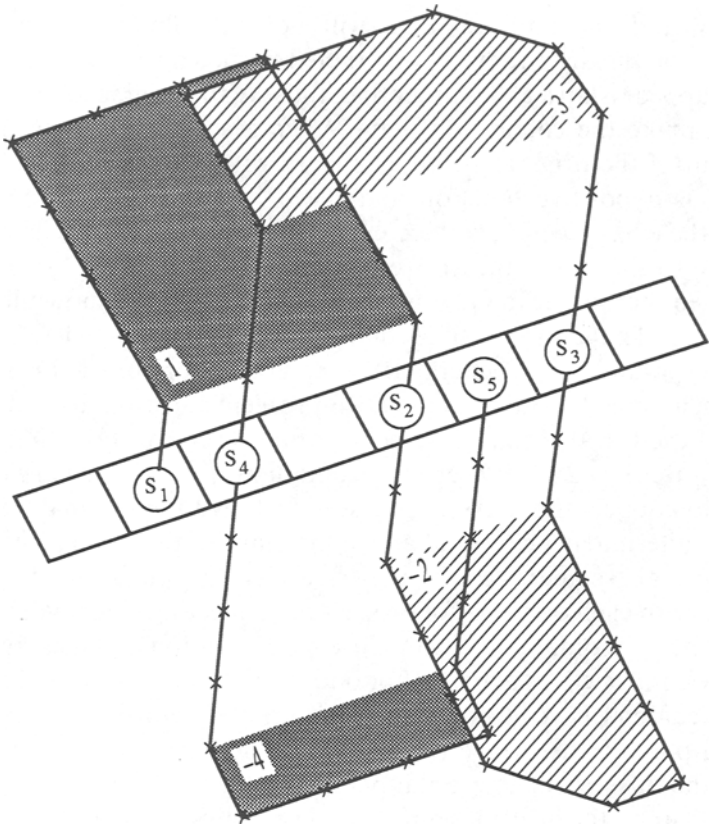

Figure 3. The transformation of the one-dimensional optimal linear arrangement problem to the three-dimensional protein folding problem. The original elements (in this example $(s_1, s_2, \ldots, s_5)$) of the OLA problem are connected by the additional xs. The connecting chain starts from s_1, goes vertically up to the horizontal plane $+1$, then leaves the vertical plane that goes through the original stripe, completes the turn by horizontal movements in plane $+1$, comes back to the point above s_2 and goes vertically down to s_2. A similar trajectory is used to connect the next pairs of elements, going alternately above and below the original stripe. Note that movements in the two additional dimensions in this way guarantees that the connecting chain will be self-avoiding. Diagonal horizontal moves are used to permit connections between elements with an odd number of cells between them.

DPF conformation is exactly equal to the original energy of the OLA instance. To see that any accepted solution for the DPF has a corresponding accepted solution for the original OLA problem we just have to note that any DPF solution which moves any of the original elements of the OLA out of the original stripe cannot be accepted as its energy is at least $B+1$. Any other accepted DPF solution (clearly, there are many such solutions) must have all the original elements on the stripe with an energy level below the limit. Thus, it is an accepted solution for the original OLA problem.

The expansion in the size of the original OLA problem when transformed to the DPF problem is polynomial, $3n+2$, and thus if the DPF problem has a polynomial solution then we have seemingly achieved a polynomial solution to the OLA problem, which is NP-complete. The only flaw is our assumption that the DPF can be solved in polynomial time, which thus must, therefore, be wrong. Thus, the DPF is shown to be as hard as the OLA NP-complete problem. As was mentioned above this implies that the underlying optimization problem "For the given model find the lowest energy folding" is NP-hard.

Discussion. Our proof is based on utilizing the function **g** to simulate the behavior of the one-dimensional, optimal linear arrangement problem on the three-dimensional lattice of the protein folding problem.

To achieve the simulation we used a discontinuous function. While we had to introduce the discontinuity as part of the formal proof, we note that discontinuity is inherent in protein folding. On the most fundamental quantum level free energy functions are not continuous. Moreover, limitations on the values of the dihedral angles in proteins (Ramakrishnan and Ramachandran, 1965) and restrictions on contiguous pairs of dihedral values (Unger *et al.*, 1990) result in making some positions in space virtually unavailable to some atoms, making the corresponding free energy function effectively discontinuous.

In our model we have used a general pairwise free energy function. One can argue that the "real" free energy function is a special case under which the problem can be solved efficiently. This is very unlikely. The most realistic free energy functions, which are based on theoretical studies, analysis of small molecules and direct experiments probing isolated components of the free energy, are complicated and don't lend themselves to simple solutions. It has been shown in molecular dynamics simulations that under these functions the free energy surface is very "rugged" and not simple (Levitt, 1983a).

Our model is a representation of real proteins. However, we note that from a theoretical point of view and for the purpose of testing search algorithms there is also interest in simpler models, for example, a two-dimensional lattice which allows only straight (non-diagonal) movements (Covell and Jernigan, 1990; Dill, 1990) and a simple energy function counting only one type of neighboring interaction. Our experience with such models suggests that finding their lowest energy is as hard as the full three-dimensional lattice. However, in the framework of the current proof we could not show that these simple models belong to the NP-complete or NP-hard classes. In our proof we use the two additional dimensions to guarantee that the connecting chains have a collision-free path. We further use the diagonal movements to enable connection between original elements regardless of the parity of their distance.

Using the three-dimensional matching problem Fraenkel (Fraenkel, 1993)

shows that a general two-dimensional model (which is extendable to three-dimensional) based on Coulomb energy minimization in NP-hard. Recently, Ngo and Marks (1992) published an NP-hard proof for a three-dimensional polymer structure prediction, using reduction from the partition problem.

The principal implication of our proof is very striking. As there is no general efficient feasible way to find the lowest free energy conformation of a protein we cannot assume that the native state is at the free energy global minimum.

The general question of the relationship between theoretical computational considerations and the actual behavior of natural systems has not been fully explored. While Church's hypothesis (Kleene, 1952) seems to bind any natural device to computational limits, it has been suggested recently that exponential computational barriers can be overcome by using natural physical systems as the core of computational devices (Fraenkel, 1990). Although one can argue about the general significance of computational complexity results to the analysis of natural systems, in the protein folding case the consequences of the proof appear unavoidable. Possible mechanisms for real protein folding seem to be transferable to computations that can be performed on a computer. The most realistic computational folding process, molecular dynamics (see for example Levitt, 1983b; and a review in Brooks *et al.*, 1988), is still restricted by the NP-hardness proof. In this model each atom is assumed to have some internal thermal energy and then is bound to move in the direction of the vector of the forces applied to it by all the other atoms in the system. To simulate a continuous behavior the forces and thus the resulting accelerations and velocities are recalculated at very short (10^{-15} sec) time intervals. This computation is of a parallel nature, but can (and is, on conventional computers) be simulated by a sequential algorithm. The speed-up of this process that the natural system can achieve is only polynomial, considering each one of the n atoms to be a separate "processor", or even each of the n^2 interactions as a separate "processor", and thus this mechanism uses a polynomial amount of resources and therefore cannot be guaranteed to find the global minimum.

Several arguments have been advanced to support the idea that the functional conformations of proteins are at the global free energy minima: Denaturation/renaturation experiments, when they work, always lead to the same fold (Anfinsen, 1973). In the few cases where proteins have been produced by peptide synthesis they have also adopted the same conformation as the biologically produced material (for example, see Wlodawer *et al.*, 1989). No protein has been found to be in more than one conformation (excluding those induced by functional changes), even when crystallized under quite different conditions (many examples can be found in the PDB data base, Bernstein *et al.*, 1977; for a specific example see Svansson *et al.*, 1991; or observed in solution by NMR, for example, Clore and Gronenborn, 1991).

Actually, these observations demonstrate that each protein has a unique functional conformation; they do not show directly that this conformation must always be the global free energy minimum. So, these observations only show that the properties of proteins are consistent with their functional conformation being at the free energy global minimum, but they do not prove it. There is another mechanism by which the same conformation can be found under a variety of conditions, in spite of the size of the conformational space, and that is by the existence of pathways from any accessible conformation to the functional minimum. That is, "entry points" to the pathway must be present everywhere in the populatable conformational space. Several pathways with this property have been proposed (Wetlaufer, 1973; Karplus and Weaver, 1976; Moult and Unger, 1991). These all depend on the notion that although the exponential search of the conformational space of the full protein is too large to be the mechanism, such a search of small fragments on an appropriate time-scale is possible. Wetlaufer argued that the maximum size of such fragments for an exponential search is *ca* 16 residues. Thus, short regions of the polypeptide chain are postulated to have preferred conformations, which although not the only populated ones early in folding, are present in high enough amounts to drive the subsequent pathway. The later events may be either propagation of the folded structure out from these folding initiation sites (Wetlaufer, 1973) or the association of such sites (Karplus and Weaver, 1976) in their folded form to produce the next level of structure. As such initiation sites are based on contiguous residues of the chain they are always accessible in the search space. In denaturation/renaturation experiments once the native conditions are restored these local interactions immediately become effective and lead the folding again towards the native structure.

We propose an evolutionary argument which strongly suggests that the functional conformation will coincide with the global free energy minimum for many proteins. We know that the functional conformations of proteins are characterized by a very high level of organization of the individual atomic interactions—they are well packed, buried polar and charged groups interact with other such groups, and non-polar cores provide much of the free energy to stabilize the structures (for a review, see Dill, 1990). To be competitive any lower free energy conformation must also exhibit these features. Random mutations of the sequence over time will tend to disrupt such intricate arrangements. For the functional conformation mutations which destabilize the structure significantly will also disrupt function and thus will not be accepted, whereas stabilizing mutations are likely to be accepted. So the free energy of the functional conformation will tend to become lower. Assume that originally the functional structure was not the global minimum, but rather a stable local minimum. Then, for the original, nonfunctional, global minimum there will be no constraints to prevent a drift upwards in free energy, so that the

conformation will in time cease to be the global minimum. Thus, eventually the functional conformation will become the one with the lowest free energy.

In conclusion we want to present our views on the different theoretical considerations of the folding process. As we see it there are three possible alternatives: (1) The "Strong thermodynamic hypothesis": Proteins are physical systems and as such reach the global energy minimum. Thus, folding is a universal process and *every* amino acid sequence (natural or random) will be folded to its global minimum. (2) The "Weak thermodynamic hypothesis": Nature has selected only proteins for which the native conformation is in the global minimum. These natural proteins have the property that for them there are more efficient ways to find the global minimum. (3) The "Kinetic hypothesis": The native conformation is largely dictated by the folding pathway and is not necessarily the global free energy minimum.

The NP-hard proof can rule out possibility (1), but it cannot help us to determine between possibilities (2) and (3). Actually, the evolutionary argument discussed above suggests a mechanism by which most current proteins have been converted from the "kinetic hypothesis" to the "weak thermodynamic hypothesis", closing the practical gap between the two possibilities. Still, there is a theoretical difference between the two possibilities. The "weak thermodynamic hypothesis" means that the folding process is a global free energy optimization process that deals with "easy" instances. The "kinetic hypothesis" means that the final conformation of a protein is determined by events along the folding pathway. The "kinetic hypothesis" is more consistent with mutation data (Serrano *et al.*, 1992) and quenched NMR data (Udgonkar and Baldwin, 1988) that demonstrate the existence of some specific pathway events during the folding process.

The important practical implication of the proof is that there is no universal algorithm which can find the global free energy minimum conformation for all given sequences. Combined with recent data on the importance of the kinetic process, it follows that algorithms that attempt to determine the conformation of proteins from the amino acid sequence should be based on the specific mechanisms that are employed by real proteins, rather than on general search methods. That is, they must in essence mimic the pathway mechanism used by proteins themselves.

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