Resistance proof, folding-inhibitor drugs

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Abstract

Conventional drugs work, as a rule, by inhibiting the enzymatic activity of specific proteins, capping their active site. In this paper we present a model of non-conventional drug design based on the inhibiting effects small peptides obtained from segments of the protein itself have on the folding ability of the system. Such peptides attach to the newly expressed (unfolded) protein and inhibit its folding, inhibition which cannot be avoided but through mutations which in any case denature the enzyme. These peptides, or their mimetic molecules, can be used as effective alternative drugs to those already available, displaying the advantage of not suffering from the upraise of resistance.
Drugs perform their activity either by activating or by inhibiting some target component of the cell. In particular, many inhibitory drugs bind to an enzyme and deplete its function by preventing the binding of the substrate. This is done by either capping the active site of the enzyme (competitive inhibition) or, binding to some other part of the enzyme, by provoking structural changes which make the enzyme unfit to bind the substrate (allosteric inhibition). The two main features that inhibitory drugs must display are efficiency and specificity. In fact, it is not sufficient that the drug binds to the substrate and reduces efficiently its activity. It is also important that it does not interfere with other cellular processes, binding only to the protein it was designed for. These features are usually accomplished designing drugs which mimick the molecular properties of the natural substrate. In fact, the pair enzyme/substrate have undergone millions of years of evolution in order to display the required features. Consequently, the more similar the drug is to the substrate, the lower is the probability that it interferes with other cellular processes. Something that this kind of inhibitory drugs are not able to do is to avoid the development of resistance, a phenomenon which is typically related to viral protein targets. Under the selective pressure of the drug, the target is often able to either mutate the amino acids at the active site or at sites controlling its conformation in such a way that the activity of the enzyme is essentially retained, while the drug is no longer able to bind to it. An important example of drug-resistance is connected with AIDS. In this case, one of the main target proteins, HIV-protease, is able to mutate its active site so as to avoid the effects of drug action within a period of time of 6-8 months (cf. e.g. [1, 2]). In the present paper we discuss the design of drugs which interfere with the folding mechanism of the target protein, destabilizing it and making it prone to proteolysis. We shall show that these drugs are efficient, specific and do not suffer from the upraise of resistance. The model of protein folding we employ is largely used in the literature. In spite of its simplicity, it reproduces well the thermodynamic and kinetic properties of real proteins [3, 4, 5]. The model describes a protein as a chain of beads sitting on a cubic lattice, each bead representing an amino acid and interacting with the neighbouring beads through a contact potential. There are twenty kinds of beads to account for the twenty kinds of natural amino acids. Consequently the contact potential is defined by a \(20 \times 20\) matrix, extracted from statistical analysis of the contacts of real proteins [6].

Using this model, it has been shown that single domain proteins fold according to a hierarchical mechanism [4, 5]. Starting from an elongated conformation, it is found that, highly
conserved and strongly interacting amino-acids lying close along the designed chain form small local elementary structures (LES). Due to the small conformational space available and to their large attractive propensity, these LES are formed at a very early stage in the folding process and are very stable. The rate limiting step of this process corresponds to the assembly of the LES to build their native, non–local contacts (folding nucleus). This nucleation can be done in a relatively short time, because LES, moving as almost rigid entities, not only reduce the conformational space available to the protein but also display low probability of forming non-native interactions. Furthermore they interact with each other more strongly than single amino acids belonging to these structures do. The nucleation event corresponds to the overcoming of the major free energy barrier found in the whole folding process. After this is accomplished the remaining conformational space available to the protein is so small that the system reaches the native state almost immediately. In keeping with these results we suggest the use of short peptides with the same sequence as the LES (in the following, shortened as p–LES) to destabilize the protein. We test this suggestion on three sequences designed to fold to the three different structures displayed in Fig. 1. The corresponding sequences are listed in the caption to the figure. It was shown in a previous work that the associated LES are built out of residues 3–6, 11–14 and 27–30 for sequence (a) of Fig. 1 (known as S36 in the literature), of residues 1–6, 20–22 and 30–31 for sequence (b) and of residues 34–42 and 2–12 for sequence (c).

To assess the ability p–LES display in destabilizing designed proteins, we have performed Monte Carlo simulations of a system composed of the protein and a number \( n_p \) of p–LES in a cubic cell of linear size \( L \) with periodic boundary conditions. Each simulation starts from a random conformation of the system and is carried on through \( 10^8 \) MC steps at fixed temperature \( T \). During the simulation, we have collected the histogram of the order parameter \( q \), defined as the relative number of native contacts, parameter which measures the extent to which the equilibrium state reached by the protein is similar to the native conformation.

In Fig. 2(a) we display the equilibrium distribution of \( q \), calculated at \( T = 0.24 \) and \( L = 7 \) for the system composed of sequence S36 and a number of p–LES 3–6 as a function of \( n_p \) (concentration). While the distribution of \( q \) values in the absence of p–LES (solid line) shows a two–peaks shape, reflecting a all-none transition between the native \((q > 0.7)\) and the unfolded \((q < 0.6)\) state, the presence of p–LES reduces markedly the stability of the
protein. The effect of the other p–LES, i.e. 11′–14′ and 27′–30′, is similar to that found for the peptide 3′–6′ and is displayed in Figs. 2(b) and (c). The strength of the inhibitory effect, measured in terms of relative population $p_1$ of the native state ($q > 0.7$) of the protein in presence of p–LES, is displayed in Fig. 3.

To further test the validity of these results, we have repeated the above calculations making use of peptides corresponding to segments of the protein sequence other than those corresponding to LES. In Fig. 2(d) the effect of peptides corresponding to residues 8–11 is shown. One can notice that the protein is not destabilized to any significant extent. To ensure that this result is not a consequence of the weak binding of the peptide to the protein, we have replaced the amino-acids 8′–11′ of the peptide by amino acids which interact with the complementary amino acids of the protein (i.e. amino acids 21,22,15 and 14 respectively, cf. Fig 1(a)) as strongly as those belonging to LES do. No difference with the results shown in Fig. 2(d) was found.

The thermodynamics which is at the basis of the disruptive mechanism of p–LES is quite simple. In fact there are three thermodynamically relevant states in the range of temperatures where the protein is stable: 1) the state in which the protein is folded and the $n_p$ p–LES do not interact with the protein (whose free energy is taken as reference and assigned a value $\Delta F_1 = 0$), 2) the state in which a p–LES is bound to the (complementary) LES of the protein preventing it from folding, its free energy being $\Delta F_2 = \Delta F_0 + E_{LES} + TS_t - T \log n_p$ where the quantity $\Delta F_0$ is the difference in free energy between the unfolded and the native state of the isolated protein, $E_{LES}$ is the interaction energy between the p–LES and the complementary LES and $S_t$ is the translational entropy of a p–LES, 3) the state in which the protein is unfolded and the p–LES do not interact with the protein, the associated free energy being $\Delta F_3 = \Delta F_0$. The translational entropy can be estimated using the relation

$$S_t(n_p) = \log \left[12 \cdot (V - v_{prot} - (n_p - 1)v_{ples})\right],$$

where $V = 343$ is the volume of the cell (in lattice units) in which the simulations are performed, $v_{prot} = 166$ is the average volume occupied by the protein, $v_{ples} = 20$ is the average volume occupied by a p–LES, while the prefactor 12 accounts for the orientation of the p–LES. It then follows that the equilibrium probability that the protein is folded, i.e.
in state 1), is given by
\[ p_1 = \frac{1}{1 + e^{-\Delta F_0/T} [n_p e^{-S_t(n_p)} - E_{LES}/T + 1]}. \] (2)

In Figs. 3(a), (b) and (c) are displayed the values of \( p_1 \) associated with the sequence S36 and the three p–LES 3’–6’, 11’–14’ and 27’–30’ as a function of \( n_p \) (solid dots). The continuous curves are the results obtained making use of Eq. (2) and of the numeric values \( \Delta F_0 = -0.038 \) (obtained from MC simulations) and \( E_{LES} (= -2.5(a), -2.0(b), -2.5(c)) \).

The overall agreement found between the three-state model and the results of MC simulations suggests that the destabilization of the protein is, in fact, due to the binding of the p–LES to the protein. Naively speaking, the protein prefers to bind the p–LES instead of the native LES because in this ways it saves internal entropy, which is not compensated by the loss of translational entropy.

The above results also suggest that the state associated with the p–LES bound to sites of the protein surface different from the LES is not relevant. This state has a relative free energy \( \Delta F_4 = E' + T S_t - T \log n_p \), where \( E' \) is the interaction energy between the p–LES and the surface of the protein. The effect of this state on the stability of the protein would be to raise the asymptotic value of \( p_1 \) for large values of \( n_p \). The fact that in none of the cases studied (cf. Figs. 3(a), (b) and (c)) the asymptotic value of \( p_1 \) is different from zero indicates that \( E' \ll E_{LES} \). In other words, the binding properties of the p–LES are highly specific. It could hardly have been different: since p–LES are identical to LES, a propensity of LES to bind some non–native part of the protein would imply the stabilization of a metastable state, something that evoultion tends to avoid.

In Fig. 3(d) we display the dependence of \( p_1 \) with temperature (solid dots) for the case of p–LES 3’–6’ and \( n_p = 2 \). The results of the simulations are well reproduced by the predictions obtained making use of Eq. (2). In these estimates the temperature dependence of \( \Delta F_0 \) has been approximated with that of the Random Energy Model \([7, 13]\). The non–monotonic behaviour of \( p_1(T) \) is a consequence of the competition between the stabilization of the native state and the decrease of the free energy of the unfolded states taking place as the temperature is lowered. At high temperatures, the state 3), which is independent on \( n_p \), becomes important, weakening the overall dependence of \( p_1 \) on \( n_p \). We have repeated the calculations described above, but this time making use of sequences obtained from S36
by introducing random point mutations in the LES 27-30. In this way we try to mimic the development of drug resistance of a viral protein. We observe two situations: I) if the protein is (upon mutation) still able to fold the scenario corresponding to Figs 1(a)-(c) is still valid, II) if the mutation denaturates the protein, the p-LES does not, essentially, bind any more to it.

We have found that also the dynamical properties of p–LES make them suitable to be used as drugs. Starting from a random conformation of the protein and of the peptides, we have calculated the probability $P(t)$ that the bond between residue 30 of the protein and 3' of any of the p–LES 3'-6' is formed as a function of time. This bond is chosen as representative of the interaction between the whole LES 27–30 and the p–LES 3'-6', the dynamics of the other bonds associated with the same LES being quite similar. The shape of the calculated probability function is well fitted by a single exponential $P(t) \sim (1 - \exp(-t/\tau'))$, where $\tau'$ is the characteristic time of bond formation. The dependence of $\tau'$ on the number $n_p$ of p–LES is displayed in Fig. 4 as a solid line, where it is compared to the average time needed for the p–LES to build the bond 30-3' with the protein after a random search in the volume of the cell, that is

$$12 \cdot \frac{(V - v_{prot} - (n_p - 1)v_{ples})}{n_p}.$$  

(3)

The result obtained making use of this relation is also displayed in Fig. 4 (dashed curve). The agreement with the result of the numerical simulations indicates that the random search is the actual mechanism which leads to the binding of the p-LES to its (complementary LES. The fact that $P(t)$ is well reproduced by a single exponential indicates furthermore that this is the only mechanism operative. In particular, this result excludes the possibility that the p–LES binds tightly to some other part of the protein. Such a scenario would produce a double- or more-fold-exponential shape of $P(t)$.

To be noted that the binding time $\tau'$ of p–LES to the protein is much shorter than the binding time of the associated native contact between LES within the protein. In particular, the result displayed Fig. 4 and associated with contact 30-3' is to be compared to the value $\tau = 1.3 \cdot 10^5$ of the native contact 30-3 [8]. The reason for this result is associated with the fact that, unlike LES, p–LES are not slowed down by the polymeric connection with the rest of the protein. A consequence of this fact is the ability p–LES have to bind to LES of the protein even if this is in its (equilibrium) native state. The p–LES can take advantage of the thermal fluctuations of the protein and make use of the fact that these fluctuations display
a recursion time (which, assuming that the system is ergodic, is equal to the mean first passage time) much longer than the time needed by p–LES to enter and disrupt the protein by binding to one of its the LES. As a matter of fact, we have calculated the distribution of $q-values$ starting from the protein in the native state, finding the same distribution as that displayed in Fig. 2.

Calculations as those described above and leading to the results displayed in Figs. 2-4 have also been carried out for the other two model proteins displayed in Fig. 1. The outcome of these calculations are, as a rule, in agreement with those found in connection with sequence S36. To be noted, however, an important difference found in connection with sequence b) (36-mer). This designed protein displays, in the folding process, three LES of length 2, 3 and 6, respectively. While the p–LES built of 6 residues inhibits folding as those described above, the other two p–LES do not. This is connected with the small size of these p–LES, which makes them quite unspecific. In fact, the probability that a p–LES binds to some part of the protein other than the target LES decreases exponentially with the number of residues involved.

We have shown that it is possible to inhibit the activity of a protein by disrupting its folding with the help of small peptides which mimick the LES of the protein. The very reason why LES make single domain proteins fold fast confers p–LES the required features to act as effective drugs, that is, efficiency and specificity. They are efficient because they bind as strongly as LES do. Since LES are responsible for the stability of the protein, their stabilization energy must be of the order of several times $kT$. These peptides are also as specific as LES are. In fact LES have evolved so as to prevent the upraise of metastable states and to avoid aggregation, aside of securing the protein to fold fast. The possibility of developing non–conventional drugs for actual situations is tantamount to being able to determine the LES for a given protein. This can be done either experimentally (e.g. making use of $\phi$–value analysis[14] or ultrafast stopped flow experiments) or extending the algorithm discussed in ref. [10] making use of a realistic force field. The resulting peptides can be used either directly as drugs, or as templates to build mimetic molecules, which eventually do not display side effects connected with digestion or allergies.

A feature which makes, in principle, these drugs quite promising as compared to conventional ones is to be found in the fact that the target protein cannot evolve through mutations to escape the drug, as happens in particular in the case of viral proteins, because
the mutation of residues in the LES would anyway lead to protein denaturation.

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FIG. 1: The three native structures used in the present calculations (two 36-mers and one 48-mer). The associated designed sequences are (a) S36≡SQKWLERGEATDGDLPVNGTYFSCKIM-ENVHPLA, (b) RASMKDKTVGIGHQLYNFEGEWCPAPDNTRVSLAI, (c) IMESQKWLCM-EPAHVTVIUTQGLGNVNCNPFGRSKIQDAYLFH.

FIG. 2: The equilibrium distribution of the order parameter $q$ of sequence (a) (cf. caption to Fig. 1) in presence of $n_p$ p–LES of kind 3’–6’ (a), 11’–14’ (b) and 27’–30’ (c), calculated at temperature $T=0.24$ in the units chosen ($RT_{room} = 0.6 \text{ kcal/mol}$). As control, a string corresponding to the residues 8–11 of the protein was also used (d).
FIG. 3: Stability $p_1$ (for $T=0.24$) of the native structure of S36 (protein (a) of Fig. 1) as a function of the number $n_p$ of p–LES of kind 3’–6’ (a), 11’–14’ (b) and 27’–30’ (c) present in the cubic cell (solid dots). The results displayed by the continuous curve was determined making use of Eq. (2) as discussed in the text.(d) The quantity $p_1$ associated with $n_p=2$ p-LES 3’-6’ as a function of temperature.

FIG. 4: The mean binding time $\tau'$ between the residue 30 of the protein and 3’ of the p–LES, as a function of the number $n_p$ of p–LES (solid line). The result of MC simulations is compared with the random search time predicted making use of Eq. (3) (dashed line).