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Genetic algorithms as a tool for helix design – computational and experimental studies on prion protein helix 1

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Summary

Evolutionary computing is a general optimization mechanism successfully implemented for a variety of numeric problems in a variety of fields, including structural biology. We here present an evolutionary approach to optimize helix stability in peptides and proteins employing the AGADIR energy function for helix stability as scoring function. With the ability to apply masks determining positions, which are to remain constant or fixed to a certain class of amino acids, our algorithm is capable of developing stable helical scaffolds containing a wide variety of structural and functional amino acid patterns. The algorithm showed good convergence behaviour in all tested cases and can be parameterized in a wide variety of ways. We have applied our algorithm for the optimization of the stability of prion protein helix 1, a structural element of the prion protein which is thought to play a crucial role in the conformational transition from the cellular to the pathogenic form of the prion protein, and which therefore poses an interesting target for pharmacological as well as genetic engineering approaches to counter the as of yet uncurable prion diseases. NMR spectroscopic investigations of selected stabilizing and destabilizing mutations found by our algorithm could demonstrate its ability to create stabilized variants of secondary structure elements.

Introduction

With increasing understanding of the forces governing protein folding and stability, as well as of the mechanisms of protein–protein-interactions, the interest in designing tailored proteins for biotechnological and medical applications is constantly growing. However, *de novo* design of proteins with *in silico* methods has to face the enormous problem of searching astronomically large sequence and conformational spaces [1]. Additionally, it could be shown that the protein design problem is *NP*-complete [2] from an algorithm theory point of view, meaning that no polynomial time algorithms to solve this problem are known yet. It is, however, possible to address several subproblems of the design problem with various computational and *in vitro* methods.

Going from a coarse to fine grained point of view, the first level of protein design is the design of sequences coarsely following a certain folding topology, in order to provide a structural scaffold to which later functionally relevant properties can be attached. Fortunately, the global fold of proteins is mostly determined by the pattern of hydrophobic and hydrophilic residues [3, 4], making it possible to mimic existing folds by

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providing a similar hydrophobicity pattern over the sequence. Thus, the generation of native-like folds is now reliably possible [5].

The stability of such folds, however, is mostly determined by hydrophobic packing interactions in the protein core [6, 7], which could be optimized effectively by computational methods in several cases [6, 8, 9]. Here, not only the nature of residues at certain positions is of relevance, but rather the achievement of an exactly defined rotamer distribution over the sequence making up the protein core [10, 11].

On a more fine-grained level, however, surface properties such as surface charge distribution, surface geometry, as well as the stability of local structural elements have to be designed to give the scaffold developed by the above-mentioned methods the desired structural and functional characteristics. Two complementary approaches can be used to reach that goal: *Positive design* tries to stabilize conformers exhibiting the desired features, while *negative design* is aimed at introducing interactions which are unfavourable for unwanted conformations. It could be shown that positive design strategies tend to be necessary for the engineering of stable structures, while negative design strategies can be used to optimize specificity and solubility at the cost of stability [12, 13].

One particularly powerful optimization strategy for the *in vitro* optimization of binding specificity, functional specificity, as well as stability, is the use of *in vitro* evolution methods, such as phage display [14, 15] or ribosome display [16]. Such methods were able to create engineered binding proteins to specific targets [17, 18], and to alter the substrate specificities of existing enzymes [19].

Evolutionary methods are however not restricted to *in vitro* application. So called *genetic algorithms* (GA) are long known as a powerful tool for a broad class of numerical optimization problems. Computer learning problems, spectral analysis, and modelling of epidemics are just a few examples. Genetic approaches also found early applications in the realm of protein design [20].

The basic concept of GA is modelled after biological evolution. Operators corresponding to mutation, crossover and replication are applied to a set of strings. These strings represent a population of genetically unique individuals. After creating an initial population, which can be viewed as a set of trial solutions to the optimization problem at hand, the mutation operator, which can be a simple bit-flip or a more complex operation, is applied individually to each string, or just to a subset of the population. The recombination operator allows information exchange between individual search trajectories, thereby creating a cooperative search. Finally, replication is bound to a so-called fitness function, a measure to evaluate the quality of each trial solution in the current population.

In this work, we apply GA to a subset of the protein design problem, namely to the stabilization of individual α-helices. Helix stability is known to be dependent on a set of different contribution factors such as intrinsic helix propensity of individual amino acids [21, 22], ion pair interaction and hydrogen bonding [23, 24] and aromatic interactions [25, 26]. Additional stabilization can be conferred by terminal capping sequences [27, 28]. A quantitative evaluation of sequences according to these stabilizing and destabilizing interactions on a purely sequential basis is available under the name AGADIR [29–34], which is based on a statistical mechanics approach modelling the helix-coil transition by using a database of energetic contributions derived from empirical data.

Materials and methods

Algorithm and software design

Our evolutionary helix optimization tool OPHELIA (Optimization of HELix propensity by AGADIR) uses AGADIR as a fitness function in order to develop sequences coding for stable helices under a given set of sequence specific restraints. OPHELIA is a classical genetic algorithm using a discrete representation of sequences in the form of ASCII-encoded strings using the standard one-letter code to represent amino acids. Population size, mutation probability and cut-off-values for truncation selection can be freely parameterized. An additional important feature is the use of masks, which restrict the possible positions for mutation, thereby allowing to improve the helicity of sequences in which certain positions are to remain fixed because they are, e.g. of importance for function or tertiary structure. Masks also allow to restrict certain positions in the

sequence to predetermined classes of amino acids, e.g. only charged, hydrophilic, hydrophobic, aromatic, small or branched amino acids (for definitions see later in the text). This allows for the design of helices with for example predetermined patterns of salt bridges or aromatic interactions. Masks also allow expert interaction to optimize the solubility or other functional parameters of interest, which may not be optimized by our chosen fitness function AGADIR, which is applied as a closed, external program separate from the genetic algorithm itself. Implementation of the algorithm was done in Perl 5.014.

NMR spectroscopy

Synthetic peptides were purchased from Jerini AG, Berlin, as lyophilized powder with TFA as counter ions. Peptide termini were protected by aminoterminal acetylation and carboxyterminal amidation. Solutions for NMR spectroscopic investigation were prepared by dissolving the peptides at 20 °C in aqueous buffer with a pH of 4.5 and containing 20 mM acetate, 0.01% sodium azide, and 10% D_2O to a final peptide concentration of 300 μM. TOCSY and NOESY (100 ms mixing time) spectra were recorded using a 800 MHz AVANCE800 spectrometer (Bruker Biospin, Rheinstetten) and analyzed using the program NDEE, as previously described [35].

Results and discussion

Unrestrained evolution

As a first test case, we investigated the free evolution of sequences of various lengths towards higher helix propensities. Unfortunately, as AGADIR does not score sequences for solubility, but only for theoretical helix propensity, unrestrained evolution leads mostly to poly-(I/L) sequences, which, albeit having a very large theoretical helix propensity of up to 99%, are not useful for practical applications due to their insolubility. Starting with sequences of 10 amino acids, in addition to the poly(I/L)-sequences, large aromatic clusters begin to appear in the final population, mostly broken apart by single M residues spaced in i , $i+3$ or i, $i+4$ patterns. The fork between poly(I/L) and polyaromatic sequences happens generally early in the trajectory. For all calculated evolutionary paths for sequence lengths of 5–16 amino acids, convergence was reached very fast, generally within 100–200 generations, starting from a randomized starting population of 100 individual genes. Calculation time per generation amounted to 1–5 min on a standard desktop PC.

As a first restraint to the free evolution of sequences, a fixed (i, $i+4$)-salt bridge was introduced. The mask applied was X(n)EXXXRX(n), where X denotes a position available for free mutation. With this restraint, the system still shows a tendency to incorporate large numbers of L and I residue, as well as the development of aromatic clusters. For longer sequences ($n>4$), however, the system showed a tendency to elongate the salt bridge network by further (i, $i+4$) spaced charge interactions. Helix content of the resulting peptides again was estimated up to 98% by AGADIR.

PrP helix 1 as a test case

Further tests were conducted on the basis of the sequence of human prion protein (huPrP) helix 1. Helix 1 is a stable secondary structure element in the context of the full length prion protein [36]. Several studies have been conducted on peptides corresponding to the helix 1 region. These investigation could prove by the analysis of circular dichroism as well as chemical shift data derived from NMR spectroscopy, that helix 1 retains its remarkable stability even as an isolated peptide in solution. [35, 37–39]. However, under aqueous buffer conditions, such peptides do not form rigid, well-defined structures, but rather dynamic, fluctuating ensembles, in which the helical conformer is in rapid conformational exchange with unfolded conformations. Nevertheless, in the time-average, the helical conformation still remains populated at about 30–40%.

Despite of the high stability of helix 1, recent models for the pathologically misfolded form of huPrP show that helix 1 has to unfold during the conformational transition and adopt an extended conformation in the misfolded isoform [40, 41]. It has therefore been hypothesized that helix 1 poses an energetic barrier for the pathological conformational transition. Stabilizing helix 1 by means of protein design could therefore be a viable way to

devise either a genetic therapy for prion diseases, which may exploit the effect of dominant negative inhibition [42], or to create livestock with an intrinsic immunity to prion diseases, such as Scrapie in sheep or BSE in cattle. AGADIR scores for helix 1 of several species range between 10.5% (human, cattle) and 16.5% (mouse), supporting the notion that a further stabilization of helix 1 might be possible.

Analysis of helix 1 in previous works pointed to a variety of structural elements important for its high stability in solution. Most notable here is a network of (i, $i+4$)-spaced charged residues forming three interdented salt bridges (D144-R148, D147-R151, R151-E152), whilst favourably interacting with the helix macro dipole, and a cluster of aromatic residues showing a remarkably persistent stacking during MD trajectories (Ziegler & Schwarzinger, unpublished results). In order to optimize the stability of helix 1 with evolutionary computing methods, an approach had to be developed to retain such features, while still improving the overall stability.

In initial trials we used a pattern of fixed amino acids at positions previously identified to be of importance for the stability of helix 1. The sequence of our test system corresponds to huPrP (140–158) (HFGSDYEDRYYRENMHRYP), which includes two short sequences flanking helix 1 (144–154). Those flanking sequences were held constant throughout this study. All calculations were performed using a starting population of 100 individuals randomized at the freely mutable positions. Single point crossover and truncation selection with a selectivity of 0.1 (only the fittest 10% of each generation where allowed to reproduce) were applied in each generation, while the system was allowed to evolve for 200 generations. All trial systems reached good convergence within this number of mutation–recombination–selection cycles.

Using the pattern HFGSDXXDRXXREXXHRYP, which in addition to the flanking sequences also holds the pattern of salt bridge forming residues conserved, an up to four-fold increase in theoretical helix propensity could be observed. Interestingly, the aromatic cluster present in the wild-type helix 1 formed again in the majority of the population. The resulting sequences, however, had a high content of bulky tryptophane residues (e.g. HFGS**DWYDRWWREWM**HRYP with a theoretical helix content of 60%), making them unlikely candidates for the incorporation into the structural context of a whole protein. Changing the pattern to HFGSXYXXXYYXXXXHRYP, only keeping the flanking sequences and the pattern of clustering aromatic residues constant, interestingly did not lead to the reappearance of salt bridge patterns in the converged population. The dominant sequences in the final population showed an extension of the aromatic cluster, interspersed with methionine residues (e.g. HFGS**WYMMWYYWYWM**HRYP, 69%). Again, the final population shows a prevalence of aromatic clusters comprised of bulky tryptophane residues and therefore does not seem suitable for application in the whole protein context.

A final test using a set of fixed amino acid positions was performed with the pattern HFGSXXXXXXXXXXXHRYP, which keeps only the flanking sequences constant and leaves the whole of helix 1 open to mutation. Interestingly, in this case we could observe the reemergence of stabilization by salt bridges, as well as the formation of the usual tryptophane-rich aromatic cluster. The most prevalent sequence in the final population was HFGS**WWQMWWDWYYR**HRYP, with a theoretical helix content of 73.6%. Interestingly, this is the only case in which M154 was changed to an arginine residue. In any other test system where this position was free to mutate, the methionine had been conserved. It is, however, not clear at this point how this position exerts its apparently crucial effect on the stability of helix 1. However, the general properties of this sequence are highly similar to the other test cases and most likely not applicable for designing an improved and stabilized prion protein. The results so far yielded sequences with a very high helix propensity, which are, however, of limited practical value indicating that rather unsupervised evolution, which lacks expert knowledge addressing structural or functional boundary conditions, is not the method of choice.

Thus, in order to reach an evolutionary path to sequences with improved helicity while still retaining the important characteristics of helix 1 and avoiding the above-mentioned problems, we extended OPHELIA to be able to allow mutations only within certain classes of amino acids. The masks used for this end make use of different amino acid similarity alphabets based on amino

acid substitution matrices [43] or on chemical similarity.

A similarity alphabet grouping amino acids into classes based on their chemical nature was used to allow only the evolution of residues forming the electrostatic interaction network of helix 1, while retaining the same charges at the same positions like in the wild-type. The pattern HFGSaXXacXXcaXXHRYP, where 'a' denotes a negative charge and 'c' denotes a positive charge, converged to a population with the dominant sequence HFG**SEWYERWWREWM**HRYP, with a theoretical helix content of 64.6%. Again, we observe the formation of a tryptophane cluster. The arginine residues of the wild-type are completely conserved and are never exchanged against other positively charged residues. The negatively charged residues of the wild-type, however, are in basically the whole population changed from aspartate to glutamate, presumably due to the higher helix propensity of glutamate and/or because of the better possibility of charge–charge interaction between Glu and Arg compared to other combinations.

If the pattern is further restrained to keep the aromatic cluster of the wild-type prion intact (pattern: HFGSarXacrrcaXXHRYP, 'a' and 'c' as above, 'r' denotes an aromatic residue), we observe basically the same effects as above. The final sequence after convergence was HFG**SEWYERWWREYM**HRYP with 64.6% helicity. Again, we observe the aspartate–glutamate exchange at the negatively charged positions and the conservation of M154. In this case, however, the resulting aromatic cluster is made up of a mixture of tryptophane and tyrosine residues, most notable being the newly introduced Y153, which, being placed in i, $i+4$-distance to the wild-type cluster, extends this aromatic cluster by one helix turn.

Finally, we created a pattern with fixed flanking sequences, allowing all positions of helix 1 to mutate within their similarity classes (HFGSaraacrrcamsHRYP, 'a', 'c', and 'r' as above, 'm': amides, 's': sulphur containing amino acids). The highest scoring result was the sequence HFG**SEWEERYYREQM**HRYP, with an AGADIR score of 31.5%. This is notably lower than the final scores for all other test cases. We again observe a preferential exchange from aspartate to glutamate, as well as the conservation of M154. In this case, however, the strong prevalence of tryptophane at the aromatic positions cannot be observed any longer. The double tyrosine motif contained in the wild-type sequence is conserved, only Y145 is exchanged for a tryptophane. The score, albeit being lower than in the other cases, still indicates a doubling of the helix content compared to the wild-type. In contrast to the other masks, the steric requirements of the substituted amino acids are closer to the wild-type, making this sequence an interesting candidate for further investigation.

Closer inspection of the development of sequence population throughout the evolutionary process, however, revealed several interesting points. For nearly all investigated patterns, significant increases of AGADIR score were observed within the first few generations. Most notably, we could identify several point mutations which double or even triple the helix content of the sequence compared to the wild-type. Single point mutations, which provide a maximum of stabilization while at the same time creating a minimum of interference with biological function or tertiary structure, are of particular interest for a potential application in gene therapy or for creating transgenic animals. The strongest increase of helix content for a single point mutation was observed for the N153W mutation, leading to the sequence HFG**SDYEDRYYRE-W-MH**RYP with a theoretical helix content of 39% compared to 10.5% in the wild-type. Double mutants, in contrast, did not achieve a comparable increase in helix propensity compared to the single mutants, the highest scoring sequence being HFG**SD-W-EDRYYRE-W-MH**RYP with 47.4% helix content as estimated by AGADIR.

Given the high increase in helix content for the N153W mutation and the high surface exposition of position 153 in context of the whole prion protein as determined from the DSSP [44], we chose this mutation as a promising candidate for experimental validation and further evaluation. We therefore investigated a peptide with this sequence by means of NMR spectroscopy. The peptide proved to be of similar, albeit slightly lower solubility as the corresponding wild-type peptide [35]. Due to the highly dynamic nature of such peptides, which are in rapid conformational exchange of the helical conformation with unfolded conformers, no defined structure for the peptides can be determined. This restriction is also

apparent from the analysis of $^{3}J(H^{\alpha}–H^{N})$ coupling constants, which has been performed for the wild-type peptide (data not shown) and which shows similar trends as observed in other peptides, adding further support for highly dynamic helix-coil transitions [38]. Analysis of α-proton chemical shifts, however, provides a well-established measure of conformational preference and the population of helical states in such ensembles [45–47]. Comparison of the α-proton chemical shifts, which have been corrected for the local effect of the mutation in the immediate neighbourhood [48, 49] to the wild-type showed a significant upfield shift of those resonances located at the point of the mutation and in the sequential neighbourhood up to 4 residues away (Figure 1). This is a clear indication of an increase in helix content compared to the wild-type [45], which could also be corroborated by analysis of the amide proton shifts (data not shown). The observed changes in α-proton chemical shift amounted to up to nearly 0.2 ppm, which indicates nearly a doubling of helix population for the region surrounding the point of mutation, which effectively translates to nearly complete population of the helical state even in the context of an isolated peptide without any long range constraints stabilizing the structure. This stabilizing effect might in part be explained by additional aromatic interactions with the existing cluster of aromatic residues in the sequence [25].

In particular, the mutation stabilizes the carboxyterminal part of the helix. While no further elongation of the helix is visible, a significant increase in the intrinsic stability can be observed for residues 153, 154, 157 and 158 in particular. A similar increase in helix content was also observed in an NMR-study of the globular domain at neutral pH [50], where the protein is also of increased stability [51]. Thus, the mutation selected by AGADIR enhances a naturally occurring, pH dependent stabilization.

To develop a negative control, we subsequently changed OPHELIA to be able to select against

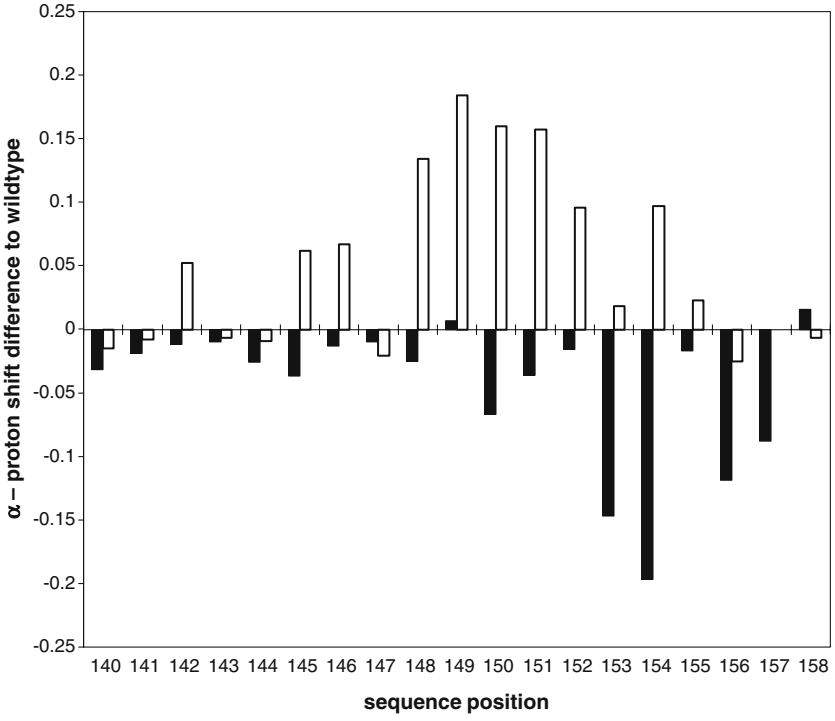

Figure 1. Graphical representation of sequence corrected chemical shift differences of huPrP (140–158) N153W (solid bars) and huPrP (140–158) R151G (empty bars) to the wild-type peptide. Negative values, as observed for N153W indicate a shift of the conformational equilibrium towards more helical conformers, whereas positive values indicate a decrease in helix population. While in both cases the maximal differences are centred at the location of the mutation, it is clearly visible that both mutation exert non-local effects on the conformation of helix 1.

increases in AGADIR score, thereby decreasing the helix propensity of sequences in the population. Again, we found significant changes in helix propensity within the first few generations of the evolutionary process. Out of those, we selected the single mutant R151G, which exhibited an AGADIR score of 2.1% compared to 10.5% in the wild-type. The effect of this mutation is rather obvious, the highly flexible glycine residue breaking up the last turn of the helix and exerting an additional destabilizing effect by disrupting the salt bridge network of helix 1. Again, the peptide corresponding to the sequence was synthesized and investigated by NMR spectroscopy. As for the N153W mutant, the expected effect could be corroborated by the experiment – R151G leads to a significant downfield shift of sequence corrected α-proton resonances, thereby indicating an destabilization of the helical conformer and a shift to a random coil ensemble [45, 48]. The destabilization is strongest in the central part of helix 1, where the highest helix content is detected in the wild-type sequence (Figure 1). Both the N153W and the R151G mutations have subsequently been introduced into the full length prion protein and are currently investigated *in vivo*, in order to test their effect on the conformational conversion of PrP.

Conclusion

We have presented a novel tool for the optimization of helix stability using an evolutionary algorithm. Evaluation of the algorithm revealed good convergence. However, initial test also showed that unrestrained evolution leads to sequences of high helix content, which, however, are of limited practical use. Therefore, a user interface allowing expert interaction was introduced. It is now possible to exclude sites that have to be conserved for structural or functional reasons. Moreover, it is also possible to restrict the optimization process to certain classes of amino acids. We have applied our algorithm to create single point mutations of helix 1 of the prion protein with maximal and with minimal helix content. The resulting mutations were not immediately obvious by intuition. Experimental verification of the selected sequences by NMR-spectroscopy underlined the effectiveness of our algorithm, demonstrating its ability to create stabilized isolated secondary structure elements. The stabilized variant N153W obtained by OPHELIA may therefore be of interest for application in gene therapy by exerting dominant negative inhibition, where expression of a stabilized prion protein variant in the background of native PrP or even a pathogenic mutation delays the onset of the disease. We propose that helix stabilization is a general strategy in the prevention of amyloidogenic diseases. Thus, OPHELIA may be applied for example to design variants of Aβ or α-synuclein variants with increased local helix propensity in order to minimize β-aggregation of these proteins. Further development of OPHELIA to incorporate negative scoring functions for selection against insoluble or aggregation-prone peptides appears to be a promising path to an even more effective sequence-driven peptide and protein design in the future.

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