Structural effects of chirally mutated Enkephalin neurotransmitters: an argument for biological homochirality.

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Abstract

In living organisms all peptides and proteins used endogenously are homochiral. However, heterochiral peptides, formed by post-translational modifications in some species, are used exclusively exogenously. These puzzling facts, accomplished at significant metabolic cost, are investigated here. A systematic study of the effect of chiral substitutions on structure and non-covalent interactions afforded by Ala(2)-Leu-Enkephalins by a combination of mass spectrometry/ion mobility spectrometry and techniques of computational chemistry is reported. The data show that chiral substitutions of amino acid residues have drastic consequences for peptide structure and their interactions as gauged by self- and cross assembly. Our data suggest that an organism based on homochiral polymers has a much higher evolvability than an organism based on heterochiral polymers.

Extant life is based on chiral polymers. That these chiral polymers are composed entirely from chiral monomer components with identical chirality in all living organisms [1], a phenomenon known as biological homochirality, has long been intensely discussed.

One point of view rationalizes biological homochirality in terms of possible scenarios as to *how mechanistically* the chemical building blocks of life's polymers may emerge in only one handedness from a presumed racemic primordial soup. To this end, numerous accidental [2–6] and deterministic [7,8] models have been proposed based on extraterrestrial [2], chemical [3–6], or physical [7,8] scenarios. However, these models provide only existential propositions of the already known fact that extant life is homochiral, and, therefore, are not testable by the scientific method [9].

Another point of view is to explain biological homochirality by saying that an organism based on heterochiral polymer systems could not exist because the processes that sustain Life would not function efficiently. In this respect, biological homochirality has been presumed essential to translation of DNA (e.g. enabling the double helical structure), to the structure/function of proteins, or to the ribosomal biosynthesis of proteins [10,11]. However, a completely achiral polymer chemistry [12,13] can yield double helical and other ordered structures, suggesting that homochirality of the nucleotides is required for *life as we know it* but not necessarily essential for *all possible life*. Further, while introducing *D*-amino acids into peptides or proteins can affect their structures significantly [14,15], recent efforts have shown that heterochiral amino acid sequences adopt a diverse range of stable folds [12,16–21], including helical and novel secondary structures, and even nanotubular aggregates [22]. Moreover, heterochiral peptides and proteins can display increased thermodynamic stability [23,24], and similar [25–27] or even significantly enhanced biological activity [28,29] compared to their homochiral congeners. These results show that heterochiral peptides or proteins are capable of taking on functional roles within an organism. Therefore, to the authors of this manuscript, the notion of impaired structure/function of

heterochiral nucleicacids, peptides, or proteins does not satisfactorily explain biological homochirality. Furthermore, recent results contest the presumption that biological homochirality is a consequence of, or a requirement for, the ribosomal translation apparatus. For one, *D*-amino acids can be aminoacylated onto tRNA by aa-tRNA synthetase enzymes [30,31]. Second, by reprogramming of the genetic code, the translation initiation apparatus was shown to accept *D*-amino acids [32]. Third, recent efforts by the Hecht [25], Suga [32], and Klussmann [33] groups showed that modified ribosomes are able to incorporate a number *D*-amino acid residues into polypeptides. It is thus conceivable that ribosomes could have evolved that incorporate *D*-amino acids into peptides or proteins. Given that the modern ribosome does not fully discriminate against amino acid chirality, it has been suggested [34] that the very early ribosomes had even significantly less chiral specificity and likely produced heterochiral peptides. In sum, contemporary structural or functional aspects of extant life's translation, transcription, or catalysis apparatus do not unequivocally support a requirement of homochirality for *all possible* life.

Indeed, Nature employs *D*-amino acids for a limited number of specific biological functions [29,35,36]. One example are the free *D*-amino acids that perform endogenous functions in the brain [37]. Other examples include small heterochiral peptides that constitute the primary bioactive ingredient in a variety of venoms [29,35,38]. Moreover, bacteria incorporate *D*-amino acids into larger structural proteins [35,37,39]. These findings underscore that heterochiral polymers can indeed take on functional roles in Nature. The authors of this manuscript are puzzled by two observations: First, these heterochiral systems are synthesized through non-ribosomal pathways or by post-translational modification of their homochiral congeners. We note that such biosynthetic routes are costly to the organism. Second, all known naturally produced heterochiral peptides or proteins are without exception applied *exogenously but not endogenously*. It appears that it can be beneficial to Nature to develop complicated and costly mechanisms to synthesize highly active heterochiral peptides and utilize them exogenously. What we

find puzzling is that Nature appears unable to use an equivalent machinery to produce heterochiral peptides that are employed *endogenously within the same organism*. The fact that heterochiral peptides outweigh their metabolic costs when utilized exogenously suggests that it is mainly endogenous homochirality that strictly matters to extant life. Why?

One specific case that is puzzling to us are the Enkephalin neurotransmitters. Enkephalins are small homochiral peptides that are used endogenously as opioid neurotransmitters in brains of mammals [40]. Heterochiral Enkephalins can adopt similar solid state structures as their homochiral congeners [41] and can display significantly greater biological activity [28], but are not used endogenously. In our opinion, the traditional accounts for homochirality do not satisfyingly explain why heterochiral Enkephalins are not used. Mechanistic models or scenarios are not satisfying because such explanations effectively state that endogenously used *Enkephalins are homochiral because they are*. Further, the heterochiral congeners $YA^D GFL^D$ and $YA^D GFL$ are biologically more active than homochiral YGGFL, excluding the explanation of impaired structure/function. Finally, even if a ribosomal synthesis of heterochiral YAGFL systems is discarded, we can't find a compelling reason why a non-ribosomal or a post-translational route to $YA^D GFL$ or $YA^D GFL^D$ should not be possible as such biosynthetic routes are available to other heterochiral peptides.

Here, we look at biological homochirality of Enkephalins from the perspective of evolvability (evolutionary adaptability), which is the capacity of an organism to generate heritable phenotype variation [42]. In a recent study [43], Lenski and co-workers demonstrated that the individual fitness of a phenotype at a given time can be less relevant to its long-term survival than its evolvability, i.e. its ability to make beneficial mutations. The question we are asking here is: does an organism with a strictly homochiral endogenous polymer basis have a greater evolvability than an organism based on heterochiral polymers?

Our argument on homochirality is based on the following four assumptions. First, we assume that *all possible* Life takes place in an environment that is subject to change. Second, we assume that *all possible* Life has to evolutionary adapt to these changes in order to sustain survival of the population. Third, we assume that *all possible* Life is based on some types of assembled polymer systems. Fourth, we assume that a large number of such polymer systems have to functionally interact with one another to sustain a living being. It is obvious that this last assumption constrains the possible combination of polymer sequences to those that are non-lethal to an organism, i.e. its *fitness*. It is less clear that this last assumptions also constrains its *evolvability*: “the greater the exactness of a polymer’s requirement for functional interaction with the other polymers, the fewer the possibilities for changes of its residues by mutation that are non-lethal” [42]. We suggest that an organism comprising a strictly homochiral polymer basis has a greater possibility of non-lethal or beneficial mutations, i.e. *evolvability*, than an organism based on heterochiral polymers.

This hypothesis is formulated on the results presented below, where we investigate effects of chiral substitutions on structure, aggregation, and biological activity of Enkephalin neurotransmitter peptides with ion mobility spectrometry/mass spectrometry (IMS/MS) and computational chemistry methods.

Material and Methods

A full description of methods and materials used is given in Supplementary Information, Section S1. For MS and IMS-MS analysis, samples were dissolved in Water:Methanol to achieve the desired concentration (100µM - 1mM). Aliquots were loaded into gold coated nanoESI capillaries, and electrosprayed to measure distributions of non-covalent peptide clusters and associated collision cross sections. In particular, to measure collision cross sections, ions are focused and stored in an ion funnel and subsequently pulsed into a drift tube filled with 13 torr of He where they are pulled through the He buffer gas under the influence of a weak electric field [44]. At the end of the drift tube ions are mass

selected and their arrival time distribution (ATD) is recorded. Ion arrival times are directly related to the collision cross-section and thus the three dimensional molecular structure of the ion (see Supplementary Information).

The gas-phase calculations for singly protonated peptides were initiated with molecular dynamics (MD) calculations with the Amber03 force field, resulting in ca 6000 candidate structures for further geometry optimization at the B3LYP/6-31G*// B3LYP/6-31+G** levels of theory using the Gaussian03 suite of programs. Explicit solvent MD calculations were carried out with the Amber03 force field in conjunction with the TIP3P water model in a truncated octahedral box and the peptide in a zwitterionic state. For all MD calculations, propagation at 300K was performed by Langevin dynamics and the SHAKE algorithm was applied.

Results

Structural and energetic properties of solvent free Enkephalins

The endogenous peptide neurotransmitter Leu-Enkephalin contains three chiral centers in its amino acid sequence YGGFL. The synthetic analogue Ala(2)-Leu-Enkephalin with sequence YAGFL contains four chiral residues that can be individually stereoinverted, resulting in three families with varying degree of heterochirality (Scheme 1).

Scheme 1

The relative energies and backbone dihedral angles ω_1 and ω_2 for the most stable solvent free backbone conformations of the Ala(2)-Leu-Enkephalin peptides studied in this work (Table S1, Supporting Information) show each enantiomeric pair as image and mirror image to each other. The two

homochiral peptides all-*L* and all-*D* YAGFL fold into a β -turn secondary structure motif with the hydrogen bonding between the C=O_{Ala(2)} and H-N_{Leu(5)} moieties (Fig. S1, Supporting Information). The Gly(3) and Phe(4) residues are located inside the turn region. This β -turn conformation with hydrogen bonding between the C=O_{Ala(2)} and H-N_{Leu(5)} moieties will in the following be denoted as a 2,5- β -turn. This structure is notably not conserved in the most stable structures of families 2 and 3 (Figs. S1 and S2, Supporting Information). Here, hydrogen bonds between the C=O_{Tyr(1)} and H-N_{Phe(4)} moieties create a β -turn conformation with the Ala(2) and Gly(3) residues inside the turn, denoted in the following as a 1,4- β -turn.

Figure 1

The structural and energetic trends induced by successive chiral substitutions are revealing when going from the all-*L* to the all-*D* geometry via families 2 and 3 (Fig. 1). Changing the chirality of either the Ala(2) or Leu(5) in all-*L* YAGFL from *L* to *D* changes the all-*L* 2,5- β -turn conformation into the 1,4- β -turn of family 2. This is a significant structural change. However, the energy increases only insignificantly ($\Delta E \sim 0.5$ kcal/mol). Chirally mutating a second residue (YA^{*D*}GFL^{*D*} from family 3) retains the 1,4- β -turn backbone conformation, but stabilizes the structure by almost 3 kcal/mol with respect to family 1. We note that a single chiral substitution of an amino acid can induce a significant conformational change into the peptide backbone without significant change in energy. Second, a single chiral substitution of an amino acid can significantly change the corresponding relative energy of a particular backbone conformation.

The peptides also adopt backbone conformations other than the respective most stable one discussed above (Fig. 1). The number of low energy backbone conformations indicates the degree of structural

heterogeneity of a given peptide. The computations indicate that the 2,5- β -turn backbone conformation is energetically favored by almost 7 kcal/mol over any other conformation for the homochiral peptides. This is a substantial gap in energy between the native and the next higher energy conformation. Significantly, no mirror image 2,5- β -turn conformation can be found for the homochiral peptides.

Several distinct backbone conformations are found for the peptide sequences $YAGFL^D$ and $Y^DA^DGF^DL$ from family 2 with chiral substitution of the Leu(5) residue (Fig. 1 and S2, Supporting Information). In addition to the 1,4- β -turn (discussed above, $\Delta E \approx 0.5$ kcal/mol), a 2,5- β -turn conformation is found only 0.2 kcal/mol higher in energy. This 2,5- β -turn conformation is structurally and energetically similar to that of the corresponding homochiral sequence (Fig. 1). However, $YAGFL^D$ and $Y^DA^DGF^DL$ can also adopt the mirror image 2,5- β -turn conformation ($\Delta E = 6.9$ kcal/mol, Fig. 1). The adoption of both mirror image 2,5- β -turn backbone conformations is also the most significant implication of stereoinverting the Ala(2) residue (YA^DGFL and $Y^DAGF^DL^D$). Here, we note an energy difference of only 2.9 kcal/mol between the two mirror images. This observation indicates a significant structural heterogeneity of the heterochiral peptides.

Changing the chirality of both the Ala(2) and Leu(5) residues creates the maximum degree of heterochirality in the YAGFL peptides (family 3). The energetically lowest conformation is a 1,4- β -turn (discussed above). Additionally, both mirror image 2,5- β -turn conformations are stable, albeit at higher energy. The most important finding here is that the energetic order of the mirror image 2,5- β -turn conformations is reversed with respect to family 2 with a ca. 7 kcal/mol difference in energy (see Table S2 and Fig. 1). This means that a single chiral substitution introduced into peptides from family 2 leads to stereoinversion of the preferred 2,5- β -turn, further highlighting the conformational heterogeneity of heterochiral peptides.

Structural properties of Enkephalins in the presence of solvent

The solvent-free calculations discussed above identified low-energy conformations exclusively as compact β -turn structures for all peptides studied due to strong hydrogen bonding between the C=O and NH moieties. However, hydrogen bonds can be disrupted by competitive solvent-peptide interactions. Consequently, solvated molecular dynamic simulations reveal extended and compact structures for the peptides investigated (Fig. S4, Supporting Information).

Figure 2

Compact structures in the presence of solvent are mostly identified as 1,4- β -turn conformations (Fig. S6, Supporting Information). Figure 2 displays the most populated compact conformations for the peptide sequences encountered in the presence of solvent when going from all-*L* YAGFL to all-*D* YAGFL by stereoinverting one residue at a time in the sequence Ala(2)→Phe(4)→Tyr(1)→Leu(5). A “flip” from the all-*L* β -turns to that of the all-*D* β -turns is observed for $Y A^D G F^D L$ (family 3).

The relative ratio of folded and unfolded structures sampled during the molecular dynamics simulations reflects the folding free energy $\Delta F_f = F_{\text{folded}} - F_{\text{unfolded}}$, i.e the free energy difference between folded and unfolded backbone conformations (Table S3, Supporting Information). The calculations indicate that ΔF_f is highly sensitive to the specific peptide sequence, and that neighboring residues with same chirality allow formation of extended, unfolded conformations. Consequently, the homochiral peptides from family 1 have the smallest propensity to fold into compact structures along with the enantiomeric pairs $Y A G F^D L / Y^D A^D G F L^D$ from family 2 and $Y^D A^D G F L / Y A G F^D L^D$ from family 3 where ΔF_f ranges from 1.7 to 2.1 kcal/mol (see Fig 3).

Figure 3

The significance of this finding is that it demonstrates that an equivalent propensity in forming extended structures can be achieved by both homochiral and heterochiral peptides. In other words: some specific combinations of chiral substitutions may appear essentially "neutral" with respect to the folding free energy. The remaining peptides with a single *L* or *D* residue from family 2 are more likely to populate compact structures ($\Delta F_f \approx 1.0$ kcal/mol). The enantiomeric pairs from family 3 with Tyr(1) and Ala(2) of different chirality display the greatest tendency to fold into compact structures in the presence of water ($\Delta F_f \approx 0.5$ kcal/mol). These data indicate that both the position and number of chiral substitutions impact the folding free energy ΔF_f significantly. We note that an increased folding free energy ΔF_f correlates on a qualitative basis with a decrease in the difference in solvation free energy $\Delta\Delta G_{\text{solv}} = \Delta G_{\text{solv}}(\text{extended}) - \Delta G_{\text{solv}}(\text{compact})$ between extended and compact backbone conformations (Fig. 3). This difference in $\Delta\Delta G_{\text{solv}}$ reflects the fact that hydrophobic residues are more shielded from solvent in the compact relative to extended structures.

Implications of chiral substitutions on interactions of Enkephalin peptides probed by ion mobility spectrometry-mass spectrometry (IMS-MS)

The results presented above indicate that structures can significantly change when amino acid residues are stereoinverted. This observation implies that chiral substitutions can affect the ability of peptides to associate and interact with other chemical components in their environment.

Figure 4

Figure 4 shows ESI-qTOF mass spectra that compare the self-oligomerisation of the two homochiral peptides all-*L* and all-*D* YAGFL to the heterochiral peptide $YA^D GFL^D$. Each spectrum shows three dominant peaks corresponding to monomer, dimer and trimer. Importantly, intermediate oligomers are amply abundant in the homochiral peptide spectra, but are greatly diminished in intensity for heterochiral $YA^D GFL^D$. This observation is also displayed in the dimer formation propensities (Fig. S9, Supporting Information). This confirms that the ability of peptides to associate and interact with other chemical components in the environment is strongly affected by chiral substitutions of their amino acid building blocks.

Figures 5 and 6

IMS-MS was used to further illustrate the effect of chiral substitutions on non-covalent interactions of the peptide systems. To this end, we compared peptide self-oligomerization in enantiomerically pure solutions to cross-oligomerization in mixtures of diastereomeric peptides with one part homochiral and one part heterochiral. Only minor shape differences for monomers and dimers are observed for the various chiral alloforms (Fig. 5). However, strong differences are observed for trimers formed in the various mixtures of diastereomeric peptides (Figs. 5 and 6). Trivially, pure all-*L* and all-*D* YAGFL display only the pure trimers $[all-L\ YAGFL]_3$ and $[all-D\ YAGFL]_3$ (Fig. 5). Mixing all-*D* YAGFL with $YA^D GFL$ (Fig. 6) results in strong prevalence of the $[all-D\ YAGFL][YA^D GFL]_2$ mixed trimer (for details, consult Supporting Information, Section S4). It is interesting to note that the equivalent trimer $[all-L\ YAGFL][YA^D GFL]_2$ is less predominant when mixing $YA^D GFL$ with all-*L* YAGFL (Fig. 6j). This observation suggests that the diastereomeric peptides all-*D* YAGFL and $YA^D GFL$ prefer to cross-oligomerize. The data further show that the interaction between the heterochiral system $YA^D GFL$ and the

two homochiral enantiomers all-*L* and all-*D* YAGFL differs as diastereomeric oligomers are formed in the mixtures. A completely different situation is encountered when changing $YA^D GFL$ to $YAGFL^D$ (Fig. 6). Now, only enantiopure trimers of each peptide (i.e. $[all-D\ YAGFL]_3$ and $[YAGFL^D]_3$) are observed. Finally, the purely heterochiral trimer (i.e. $[YA^D GFL^D]_3$) suddenly prevails when mutating both the Ala(2) and Leu(5) residues to $YA^D GFL^D$ (Fig. 6). The mixed trimer $[all-D\ YAGFL][YA^D GFL^D]_2$ is less abundant, but the purely homochiral trimer $[all-D\ YAGFL]_3$ is seen only as trailing signal. Overall, the data show that the ability of peptides to interact is greatly affected by chiral substitutions. $YAGFL^D$ interacts only weakly with all-*D* YAGFL. Strong interactions are observed for all-*D* YAGFL with $YA^D GFL$ and $YA^D GFL^D$, but the abundance, composition and structure of trimers formed differ greatly. The similarity of $YA^D GFL$ and $YA^D GFL^D$ monomer structures (Figs. S4 and S5, Supporting Information) does not explain the observed grossly different cross-oligomerization with all-*D* YAGFL. This observation indicates that chiral substitutions of peptide sequences can drastically alter interactions with other chemical components.

Structure - opioid activity relationship of Enkephalins

The structural basis of Enkephalin opioid activity has long been investigated [28,40,45]. Enkephalins were reported to lack a well-defined secondary structure in aqueous solution [45,46], in agreement with the results presented here. Determining a quantitative structure-activity relationship has so far remained insurmountable [45,46].

Figure 7

Fig. 7 shows that the *vas deferens* activity of Ala(2)-Leu-Enkephalins [28] increases exponentially with the tendency to adopt compact conformations, suggesting that folding into a compact conformation determines opioid activity. The only exception to this trend is the peptide YA^DGF^DL , which displays only minor opioid activity even though compact solution phase conformations dominate (Fig. S4, Supporting Information). The active peptides YGGFL, YA^DGFL and YA^DGFL^D adopt a common compact conformation ($\omega_1 \approx -50^\circ$ and $\omega_2 \approx 90^\circ$, Figs. S6, S8 and Table S3, Supporting Information) that differs strongly from that adopted by the inactive peptide YA^DGF^DL ($\omega_1 \approx -6^\circ$ and $\omega_2 \approx -27^\circ$). This observation suggests that the bioactive conformation of Leu-Enkephalin neurotransmitter peptides is a compact 1,4- β -turn backbone conformation with dihedral angles $\omega_1 \approx -50^\circ$ $\omega_2 \approx 90^\circ$.

Discussion

The results presented above show a strong structural heterogeneity of heterochiral Ala(2)-Leu-Enkephalins, in contrast to the homochiral congeners. In the absence of solvent, the heterochiral peptides adopt 1,4- β -turns in addition to right- and left-handed 2,5- β -turns, while the homochiral Ala(2)-Leu-Enkephalins adopt either a left- or a right-handed 2,5- β -turn. In the presence of solvent, the homochiral peptides favor extended backbone structures while no clear trend is obvious for the heterochiral sequences. Nevertheless, the data on opioid activity of the Enkephalin sequences [28] underscore that the heterochiral Enkephalins YA^DGFL and YA^DGFL^D are functionally more active than their homochiral congeners (Fig. 7). Further, our computational analysis indicates that the active peptides YGGFL, YA^DGFL , and YA^DGFL^D adopt a common biologically active conformation. Our computational analysis is supported by crystallographic data [41] that show similar solid state structures for homochiral YGGFL and heterochiral YA^DGFL^D (see Fig. S10, Supporting Information). The ion mobility data show that the propensity of a peptide to non-covalently interact with others changes

strongly when its residues are successively stereoinverted (see Fig. 6). This observation is significant because it indicates that chiral mutagenesis would lead to drastic changes in non-covalent interactions between various compounds in an organism.

Post-translational or non-ribosomal synthesis of heterochiral peptides is common for certain peptides [29] but is not done in the Enkephalin case. This would appear reasonable in the light of traditional accounts of homochirality only if homochiral Leu-Enkephalin differs in structure from the more active heterochiral Ala(2)-Enkephalin sequences. Yet, the data presented in this work indicate a common bioactive structure for endogenous YGGFL and the more effective heterochiral peptides $YA^D GFL$ and $YA^D GFL^D$ (Fig S7, Supporting Information). In fact, there are no energetic or biochemical [25,32,33] aspects that would argue against biosynthetic access to an endogenous heterochiral peptide such as $YA^D GFL^D$, but heterochiral peptides/proteins are not used for endogenous function. Therefore, in consideration of the implications of amino acid chirality on structure, aggregation and opioid activity of Enkephalin peptides presented in the current work, we suggest that homochiral YGGFL may be considered a “compromise peptide” that is optimized to mimic structural and functional features of the more effective heterochiral opioid peptide $YA^D GFL^D$ within the constraint of endogenous homochirality. This notion is supported by recent investigation of a bicyclic peptide inhibitor of the cancer-related protease urokinase-type plasminogen activator (uPA) [47]. The study found that mutating a glycine residue with positive ϕ dihedral angle to *D*-serine increased biological activity of the peptide. Further, the authors indicated that glycine residues with positive ϕ dihedral angles can generally be replaced by *D*-amino acids without compromise in biological function. In sum, it appears that life benefits from a homochiral biopolymer basis even though specific endogenous processes may have to proceed less effectively than they ultimately could in a heterochiral world.

Living systems are required to adapt to a constantly changing environment. Consequently, evolutionary changes of the kind that supports life are a necessity for sustainable life. Therefore, successful evolutionary response of life to changes in the environment requires a molecular machinery that is finely tuned to provide functional efficiency during the life span of an individual being (i.e. *fitness*) and at the same time functional stability to evolutionary adapt to a changing environment (i.e. *evolvability*). In the context of evolvability, we emphasize that “the greater the number and exactness of a polymer’s requirement for functional interaction with the other polymers, the fewer the possibilities for changes of its residues by mutation” [42]. Our data on the implications of amino acid chirality on structure and non-covalent interactions of Enkephalin peptides presented in the current work demonstrate that intermolecular interactions afforded by proteins/peptides change significantly if chiral mutagenesis were allowed. Therefore, we infer from our data that random chiral mutations of peptide/protein residues would likely have drastic implications on their function when traits are passed on to offspring in the context of evolution. Consequently, we conclude that our data suggest that the evolvability of an organism with a homochiral endogenous polymer basis is greater than that of an organism which endogenously uses heterochiral polymers. Thus, in the context of the findings reported by Lenski and co-workers [43], we postulate that heterochiral Enkephalin neurotransmitters are not naturally employed because Nature has optimized evolvability through using an endogenous homochiral polymer basis at the cost of the effectiveness of individual endogenous chemical processes. Findings in biopolymer chemistry and synthetic biology support this view. Without participating in the discussion of the importance of genetic drift and positive or negative selection to evolution [48], it is noted that natural (homochiral) biopolymers commonly display a remarkable structural and functional stability against substitutions in their sequence [49,50], which would not be possible in a heterochiral polymer basis.

Conclusions

Our data suggest that an organism based on homochiral polymers has a higher evolvability than an organism based on heterochiral polymers. Given that no observations point to energetic, structural, or functional reasons why *D*-amino acid residues should not be incorporated into proteins, we postulate that a structural and functional stability against substitutions in polymer sequence is an important property of the molecules of life and that this stability on an evolutionary time scale can only be achieved with a homochiral polymer basis.

Thus, we conclude that the overwhelming degree of homochirality displayed in biological systems is necessary because life could exist on a heterochiral polymer basis only in a form in which traits are passed on from ancestor to offspring free of error, i.e. without evolution. However, living systems are required to adapt to a continuously changing environment. Therefore, an endogenous homochiral polymer basis is needed for life to effectively sustain evolutionary response to changes in the environment. For this reason we speculate that wherever Life exists, the molecules supporting Life will be homochiral.

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Author Contributions

C.B., N.F.D and M.M.G. made the measurements. C.B. performed the calculations and C.B., N.F.D. and M.T.B. designed the study. C.B. and M.T.B. co-wrote the manuscript.

Legends to the Schemes and Figures.

Scheme 1.

YAGFL contains four chiral residues that can be individually stereoinverted, resulting in three families with varying degree of heterochirality. Family 1 consists of the two homochiral peptides all-*L* and all-*D* YAGFL. Family 2 contains the eight peptides with a single chiral substitution with respect to the all-*L* and all-*D* forms. Family 3 is comprised of six peptide sequences in which two residues with *L* configuration are mixed with two residues of *D* configuration. The five backbone α -carbon atoms define two dihedral angles $\omega_1 \angle(C_{\alpha, \text{Tyr}}, C_{\alpha, \text{Ala}}, C_{\alpha, \text{Gly}}, C_{\alpha, \text{Phe}})$ and $\omega_2 \angle(C_{\alpha, \text{Ala}}, C_{\alpha, \text{Gly}}, C_{\alpha, \text{Phe}}, C_{\alpha, \text{Leu}})$ that characterize the topology of the peptide backbone.

Figure 1. Relative energies of Ala(2)-Leu-Enkephalin gas-phase conformations as a function of their *D*-amino acid content resembling the all-*L* and all-*D* 2,5- β -turn (red and green, respectively) and 1,4- β -turn conformations of YA^DGFL (blue). Changing the chirality of either the Ala(2) or Leu(5) in all-*L* YAGFL from *L* to *D* changes the all-*L* 2,5- β -turn conformation into the 1,4- β -turn of family 2. This is a significant structural change without significant change in energy ($\Delta E \sim 0.5$ kcal/mol). Chirally mutating a second residue (YA^DGFL^D from family 3) retains the 1,4- β -turn backbone conformation, but stabilizes the structure significantly (~ 3 kcal/mol). Simultaneously changing the chirality of four residues (YA^DGFL^D to $\text{Y}^D\text{AGF}^D\text{L}$) then changes the structure to its mirror image, with subsequent chiral substitutions resulting in the fold of all-*D* YAGFL. Significantly, heterochiral peptides are able to adopt both mirror image 2,5- β -turn conformations.

Figure 2. Most populated compact structures of the all-*L* YAGFL to all-*D* YAGFL when sequentially mutating the chirality of the residues Ala(2), Phe(4), Tyr(1) and Leu(5). It is seen how the backbone fold changes from the all-*L* to the all-*D* conformation. The largest changes in conformation occur when one of the internal chiral residues Ala(2) and Phe(4) is mutated. As was the case in the gas-phase calculations, a “flip” from the all-*L* β -turns to that of the all-*D* β -turns for the two-fold chirally mutated sequences from family 3 is observed. The backbone is indicated in solid black for clarity.

Figure 3. Relationship between the folding free energy ΔF_f and the computed difference in solvation free enthalpy $\Delta\Delta G_{\text{solv}}$ between compact and extended structures. A linear regression (filled squares) is indicated in the figure. Outliers not included in the regression (filled circles) belong to sequences $\text{YAGF}^{\text{D}}\text{L}/\text{Y}^{\text{D}}\text{A}^{\text{D}}\text{GFL}^{\text{D}}$ and $\text{Y}^{\text{D}}\text{A}^{\text{D}}\text{GFL}/\text{YAGF}^{\text{D}}\text{L}^{\text{D}}$ from families 2 and 3, respectively.

Figure 4. ESI-qTOF spectra for sequences (a) all-*L* YAGFL, (b) $\text{YA}^{\text{D}}\text{GFL}^{\text{D}}$, and (c) all-*D* YAGFL. Peaks are annotated with their n/z values where n is the oligomer number (monomer=1, dimer=2, etc) and z is the charge. Note that the oligomer abundances are essentially identical for the two homochiral sequences from family 1. The homochiral peptides have a stronger propensity for self-assembly than $\text{YA}^{\text{D}}\text{GFL}^{\text{D}}$. In particular, large oligomers of up to 20-30 monomer units are readily identified for the homochiral sequences in the blow-up of the m/z 2500 to m/z 4000 range, while a nonamer is the largest oligomer that can be identified in that mass range for $\text{YA}^{\text{D}}\text{GFL}^{\text{D}}$. Please note that each solution is enantiomerically pure.

Figure 5. ATDs recorded on the ESI-Q instrument of $n/z = 1/1$ and $3/2$ of YAGFL, all-*D* YAGFL, $\text{YA}^{\text{D}}\text{GFL}$, YAGFL^{D} , and $\text{YA}^{\text{D}}\text{GFL}^{\text{D}}$. The cross sections and assignments of features within each ATD

are noted, with solid circles representing homo-chiral YAGFL (all-*L* or all-*D*) monomers and open circles representing the corresponding hetero-chiral monomers that exist within each oligomer. The 1/1 data show monomer peaks and dimer peaks (as indicated by one and two circles); the 3/2 data contain trimers (indicated by three circles). The traces (light gray) are the theoretical peak shapes calculated from equation S1.5. Note how close the experimental and theoretical peak shapes are indicating a very narrow range of variation in the various structural families.

Figure 6. ATDs of $n/z = 1/1$ (a-h) and $3/2$ (i-p) of various chiral mixtures in 1:1 ratio. All-*L* YAGFL or all-*D* YAGFL was mixed with YA^D GFL, $YAGFL^D$, or YA^D GFL D . The cross sections and assignments of features within each ATD are noted, with solid circles representing homo-chiral YAGFL (all-*L* or all-*D*) monomers and open circles representing the corresponding hetero-chiral monomers that exist within each oligomer. Cases where the homo-chiral and hetero-chiral peaks overlap are indicated with /. The 1/1 data show monomer peaks and dimer peaks (as indicated by one and two circles); the 3/2 data contain pure homochiral and pure heterochiral trimers, as well as mixed trimers, with both homo- and hetero-chiral monomers within a single assembly. ATDs of homochiral YAGFL are identical to those shown in Figure 5 and are included for comparison. The traces (light gray) are the theoretical peak shapes for a single structure calculated from equation S1.5. Note all monomers, dimers and homochiral trimers show good agreement for a single structure. The mixed trimers however, have broader experimental peak shapes indicating multiple conformations in the structural families. Spectra were recorded on the ESI-Q instrument.

Figure 7. Correlation between the measured *vas deferens* activity α of naturally occurring Leu-Enkephalin (YGGFL) and Ala(2)-Leu-Enkephalin analogs and the computed folding free energy

ΔF . The opioid activity[28] increases exponentially with decreasing folding free energy ΔF (Table S3, Supporting Information), i.e. the tendency of the peptide to adopt compact conformations. This result suggests that folding into a compact conformation that then binds to the receptor determines opioid activity. The exception to this is the sequence YA^DGF^DL , which displays limited opioid activity although compact conformations dominate. The active peptides YGGFL, YA^DGFL and YA^DGFL^D adopt a common compact conformation ($\omega_1 \approx -50^\circ$ and $\omega_2 \approx 90^\circ$) that differs strongly from that of the inactive peptide YA^DGF^DL ($\omega_1 \approx -6^\circ$ and $\omega_2 \approx -27^\circ$). This result suggests that the bioactive conformation of Leu-Enkephalin neurotransmitter peptides is the compact 1,4- β -turn backbone conformation common to YGGFL, YA^DGFL and YA^DGFL^D . (Note that the average ΔF of the two corresponding enantiomers is plotted in the Figure to minimize fluctuations due to the finite time length of the molecular dynamics simulations.)

Scheme 1.

$$\omega_1 = \angle(C_{\alpha, \text{Tyr}}, C_{\alpha, \text{Ala}}, C_{\alpha, \text{Gly}}, C_{\alpha, \text{Phe}})$$

$$\omega_2 = \angle(C_{\alpha, \text{Ala}}, C_{\alpha, \text{Gly}}, C_{\alpha, \text{Phe}}, C_{\alpha, \text{Leu}})$$

Figure 1.

Figure 2.

The figures indicate folded, solvated structures

Figure 3

Figure 4.

Figure 5

Figure 6

Figure 7.

H-Tyr-Ala-Gly-Phe-Leu-OH

Dimer/Monomer
ratio

D-amino acid content [%]