Differential hydration of enantiomers may have played a role in the selection of L-amino acids by early forms of life. Enhancement can be induced by mechanical agitation, stirring, catalytic processes of crystallization, such as an enantiomeric enhancement. Features of synthetic D- and L-polyglutamic acid and polylysine molecules each of 24 identical groups are arranged in a unique set of spines which are chemically identical. Recent studies, however, have indicated that parity violation by the nuclear weak force induces a tiny energy difference between chiral isomers. Upon combination with a massive amplification process, expansion of this difference to a detectable macroscopic level may be achieved. Yet, experimental tests of this possibility, where one enantiomer is compared to the other in solution, are hampered by the possible presence of undetectable impurities. In this study we have overcome this problem by comparing structural and dynamic differences were by and large abolished in deuterium oxide. Our findings suggest that deviation from physical invariance between the D- and L-polyamino acids is induced in part by different hydration in water which is eliminated in deuterium oxide. Based on the recent findings by Tikhonov and Volkov (V. I. Tikhonov and A. A. Volkov, Science 2002, 296, 2363) we suggest that ortho-H₂O, which constitutes 75% of bulk H₂O, has a preferential affinity to L-enantiomers. Differential hydration of enantiomers may have played a role in the selection of L-amino acids by early forms of life.

Introduction

Space symmetry of physical laws asserts that in any macroscopic chemical or physical reaction, where achiral molecules are converted to chiral products, the system as a whole remains absolutely racemic. However, when an autocatalytic arm is associated with such a process, a slight excess of one enantiomer can be expanded considerably.1–3 In the autocatalytic process of crystallization, such an enantiomeric enhancement can be induced by mechanical agitation, stirring, or β-irradiation.6 Furthermore, it is now widely accepted that chiral isomers are inherently at a slightly different energy state due to the parity violation of the electro-weak nuclear force (parity violation energy difference, PVED). The tiny excess of one enantiomer in a racemic mixture due to PVED can, in principle, be amplified by an external autocatalytic process to a level of detectable macroscopic difference.7,8 Whether such a process could explain the initial selection which led to the homochirality of amino acids, saccharides and nucleic acids in the biological realm, remains uncertain.9,10

The above possibility was previously addressed by us in systems of supersaturated solutions of D- and L-tyrosine. D-tyrosine reached a saturated solution of lower concentration than L-tyrosine.11,12 Furthermore, crystallization of supersaturated DL-tyrosine in water led to enantiomeric enhancement of L-tyrosine in the saturated aqueous layer.11,12 Unexpectedly, the difference in the rate and level of crystallization between D- and L-tyrosine was almost fully abolished in D₂O solutions.12 We interpreted these findings by proposing a dual function of PVED. In the first, it provides a slight excess of L-tyrosine which is amplified in the crystallization autocatalytic process, while in the other it induces a preferential interaction of ortho-H₂O, which constitutes 75% of the bulk, with the L-enantiomer.12 Namely L-tyrosine is slightly more hydrophilic than D-tyrosine in H₂O, but much less so in D₂O, where spin isomers are of a much lower distinction.13

In an earlier study,14 similar trends were observed. Micelles of N-palmitoyl or N-stearoyl D- or L-serine in water displayed intense CD bands of opposite directions which are abolished by disruption of the micelles with ethanol. These specific CD bands were attributed to chiral surfaces where the serine head groups are arranged in a unique set of spines which are
integrated to a chiral micellar surface. However, the absolute level of the CD band in the D-serine micelles was about 50% stronger than that of the enantiomeric L-serine micelles. This difference indicated a tighter correspondence between the residues on the surface of the D-serine micelles due to a lesser interaction with the surrounding aqueous layer.

Experimental verification of the intriguing assertion that enantiomers, like natural amino acids, are not fully identical, is hampered by the possibility that when comparing bulk systems the presence of impurities, even in undetectable concentrations, can tip the apparent macroscopic balance to an erroneous level. In the following study we have overruled this notorious problem by comparing structures and their transitions of two separate systems of water soluble enantiomeric polypeptides: poly L- and poly D-glutamic acid and poly L- and poly D-lysine, each of 24 identical residues. In these polypeptides α-helical and random coil configurations and their transitions involve intramolecular autocatalytic processes where low level of impurities in the solvent can be considered as negligible.

Polyglutamic acid and polyllysine are water soluble polypeptides which undergo structural changes related to the degree of ionization of their side chains. When in the ionized state, these polypeptides are at an equilibrium among fluctuating unstructured conformations, generally termed as "random coil". In the neutral state they assume a well-defined structure, referred to as "random coil". In the neutral state they assume a well-folded structure, generally termed as "random coil".

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Experimental

Peptide synthesis and analysis

Synthesis of L-(Lys)24-amide, D-(Lys)24-amide, L-(Glu)24-amide and D-(Glu)24-amide were carried out on ADVANCED CHEMTECH APEX 393 multiple peptide synthesizer (Louisville, KY), employing the α-N-fluorenylmethoxycarbonyl (Fmoc) strategy, following the available commercial protocols of the company. Peptide chain assembly was conducted on Rink Amide resin (Novabiochem Lauflingen, Switzerland). Fmoc-L-Lys(Boc)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-L-Glu (Boc)-OH and D-Glu(Boc)-OH (Novabiochem, Lauflingen) served as building blocks. The overall purity of these compounds was >98%, yet over 90% of the impurities were decomposed inactive materials, as assessed by HPLC. Opposite enantiomeric active residues were undetectable by the vendor and were therefore considered as negligible.

Coupling of amino acid units was achieved by using, at each step, 4 equivalents of Fmoc-amino acid with 4 equivalents of benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP). Following detachment from the polymer support and concomitant deprotection, by acidolysis with trifluoroacetic acid (TFA). Crude oligopeptides were purified to homogeneity by reversed phase HPLC on a semi-preparative silica C-18 column (250 × 10 mm; VYDAC). Elution was accomplished by a linear gradient established between 0.1% TFA in water and 0.1% TFA in 70% acetonitrile in water (v/v). The products were collected by lyophilization and analysed for purity on an analytical C-18 column (VYDAC) and on pre-packed Chromolith Performance RP-18e column (4.6 × 100 mm; Merck, Darmstadt) using the above linear gradient. HPLC-efluents were monitored at 220 nm. Molecular weights were ascertained by mass spectrometry (VG Tofspec; Laser Desorption Mass Spectrometry; Fison instruments, Manchester, UK). For all synthesized polypeptide used in this study the recorded mass spectrometry values were within 0.2% of the calculated molecular weight. Based on our analyses, we estimated intramolecular purity be higher than 99% for all four polypeptides.

Synthetic poly-D-glutamic acid, poly-D-lysine and poly-D-alanine were obtained from Sigma and were used without further purification.

Circular dichroism (CD)

CD measurements were performed in a 1 mm quartz cuvette, with Aviv CD spectrometer model 202, at 25 °C. Each scanning was repeated at least five consecutive times and averaged. Background scanning was recorded in parallel with the solvent alone and subtracted. Concentrations of the polypeptides were determined by optical density measurements at 212 nm in a 1 mm quartz cuvette taking an extinction coefficient of 1700 M⁻¹ cm⁻¹. The final pH of the solutions was adjusted with 0.01 M H₃PO₄, 0.01 M Na₃PO₄ and 0.1 M NaOH.

Isothermal titration calorimetry (ITC)

Titration calorimetry measurements, at 30 °C, were performed with a VP-ITC calorimeter (Microcal Northampton, MA). The reaction cell (1.4301 ml) was filled with degassed Polyl or D glutamic acid or lysine solution, and the injection-stirrer syringe (0.289 ml) was loaded with 25 mM HCl solution or 100 mM NaOH solution. The concentration of the poly peptides in different experiments was in the range of 1.2–1.8 mM, taking care at each specific experiment to use essentially identical concentrations of the l and D samples. The concentration of the polymer solution was measured at 212 nm, using ε = 1700 M⁻¹ cm⁻¹. In one set of experiments the solvent (cell and syringe) was water, and in a second set of experiments the solvent was 50% D₂O – 20% H₂O (v/v). Aliquots of the injectant, in 5 µl increments, were injected into the cell, and the heat flow was measured. The titration experiment consisted of 45 injections. The duration of an injection was 10 s, with 180 s equilibration time allowed between two consecutive injections.

Data analysis

Data analysis was carried out with ORIGIN 5.0 software (Microcal).

Results and discussion

Circular dichroism

The CD spectrum of the right handed α-helix of poly-L-glutamic acid or poly-L-lysine is expected to be a mirror image of the spectrum of the left handed helices of their D-polypeptide under the same conditions. Furthermore, the energetics associated with helix formation or breaking is assumed to be
identical for any pair of polypeptides of identical size composed of the d or the l enantiomers. However, parity violation energy differences (PVED) between chiral enantiomers, which are extremely small (\( \sim 10^{-17} \) eV), can in principle be increased to a detectable level when associated with an amplifying mechanism. Helix formation in such polypeptides is a typical autocatalytic process, where each turn enhances propagation to the next turn and beyond as the helix builds up. The energetics associated with helix formation in enantiomeric polypeptides, such as poly-l- or poly-d-glutamic acid, may be thus slightly different, a possibility which has far reaching implications.

Poly-l-glutamic acid, poly-d-glutamic acid, poly-l-lysine and poly-d-lysine, each blocked at the carboxylic terminus as amide, and each of precisely 24 monomers [poly (l-Glu)\(_{24}\), poly (d-Glu)\(_{24}\), poly (l-Lys)\(_{24}\), and poly (d-Lys)\(_{24}\), respectively], were synthesized by solid phase stepwise addition of monomers, then isolated by preparative HPLC and analyzed (see Experimental). Their purity was the highest we could achieve. The precise molecular weight recorded for the polypeptides and the absence of opposite enantiomeric residues in the starting materials, led us to assess a purity of \( >99\% \). The CD spectra of poly (l-Glu)\(_{24}\) and poly (d-Glu)\(_{24}\) at their \( \alpha \)-helix (measured at pH 2.5) and random-coil (measured at pH 10.5) configurations are presented in Figs. 1 and 2. As shown, the CD spectra in Fig. 1 are mirror images typical of right handed and left handed \( \alpha \)-helices, respectively. Similar CD mirror image spectra were also recorded with poly (l-lys)\(_{24}\) and poly (d-lys)\(_{24}\) in their helix region (recorded in 0.1 M NaOH, not shown). The identity of the absolute CD bands further supported the assessment of high purity of the studied polypeptides since any presence of a contaminant in the polypeptide matrix is expected to induce a marked effect on the helix formation reflected in the CD band. The CD spectra of the helices in H\(_2\)O were stable for approximately 30 min and then gradually declined, probably due to aggregation and precipitation.

In the random coil region of both sets of polypeptides, the CD spectra of the enantiomeric couples were not identical mirror images, as presented for the polyglutamic acid set in Fig. 2, indicating a net difference in the equilibrium state of their random coil conformations. As shown in Fig. 2, D\(_2\)O markedly affected the CD spectrum of poly (l-Glu)\(_{24}\) but had a significantly smaller effect on the spectrum of poly (d-Glu)\(_{24}\). It is reasonable to assume that in this region, small differences in energy of the fluctuating conformations, which determine the equilibrium, could account for the observed deviation from mirror image spectra in the random coil region of poly (d-Glu)\(_{24}\) and poly (l-Glu)\(_{24}\). In the \( \alpha \)-helix region, on the other hand, the energies associated with the hydration and the intramolecular hydrogen bonding are presumably much larger and could mask small energy differences between these peptides which appear to be identical in their CD spectra.

**Isothermal titration calorimetry**

The above suggestion could be tested by comparing the relative transition energies between \( \alpha \)-helical and random coil configurations. We therefore conducted a detailed energetic determination of the helix-to-coil transition in these sets of polypeptides under isothermal conditions. Isothermal titration calorimetry (ITC) profiles at increments of decreasing pH were determined at 30 °C, either in H\(_2\)O or in a 4:1 (v/v) mixture of D\(_2\)O and H\(_2\)O. A summary of the data derived from the ITC profiles of poly(l-Glu)\(_{24}\) and poly(d-Glu)\(_{24}\) is presented in Table 1 and typical profiles are displayed in Fig. 3. The ITC profiles in Fig. 3 can be divided into three distinct regions related to the degree of ionization of the glutamic acid side chains: pH > 6, where the polypeptides retain an equilibrium among random coiled structures, pH \( \sim 6–3 \), the range
of transition to α-helix, and pH < 3, where the polypeptides are at their α-helix conformation. This classification was verified by CD scanning in a series of buffers of pH 2.5–10 which indicated α-helix-to-coil transition at pH B 6 for both polypeptides (not shown).

In the α-helix region, the ITC profiles indicated marginal enthalpy change for both polypeptides. Similar ITC profiles of low enthalpy change were observed for acetic acid and propionic acid in water in the pH range of 2–8 (not shown), which suggests that the carboxylic side chains of poly(L-Glu)24 and poly(D-Glu)24 behave as isolated monomers at their α-helix region. On the other hand, in the random coil region (pH > 6) complex ITC profiles were recorded for both polypeptides, which indicated correspondence in enthalpy changes between the polypeptide backbone and the carboxylic head groups. The ITC profiles of the two enantiomeric polypeptides in this pH region were similar in shape but not identical (see Fig. 3), in agreement with the structural differences recorded by CD in this region (see Fig. 2).

The most pronounced differences between the ITC profiles of poly(L-Glu)24 and poly(D-Glu)24 in H2O were recorded in the transition region. The onset of the transition of poly (D-Glu)24 was 0.2–0.3 pH units above that of poly (L-Glu)24, namely, at a lower level of protonated side chains (see Table 1). This transition point is independent on concentration. In other words, the transition to α-helix of poly (D-Glu)24 started at a point of higher proportion of ionized side chains than in poly(L-Glu)24 (at pH 6.2 compared to 5.8, respectively), indicating a stronger tendency of poly-D-glutamic acid to adopt an α-helix structure. Both the degree of cooperativity and the associated change in enthalpy of the transition to α-helix of poly(D-Glu)24 were considerably higher than that of poly (L-Glu)24 (see Table 1). It is important to stress, that the abolition of the differences described above between the enantiomeric polypeptides in water by D2O overrules the possibility of an undetectable flaw in their synthesis. Under such a hypothetical case the results in both solvents would be identical.

The most plausible interpretation for the above differences is that poly(D-Glu)24 has a higher α-helix stability than poly (L-Glu)24. According to this interpretation, each residue of

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<th>Table 1 Summary of ITC results for titration of poly (L-Glu)24 and poly (D-Glu)24 with HCl</th>
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<td>In H2O</td>
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<td>Cooperativity index</td>
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<td>In mixture (D2O–H2O = 4 : 1 v/v)</td>
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<td>Mean (n = 2)</td>
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Derivation of cooperativity index is based on normalization, where the value of non-cooperativity equals 1.0 (see ref. 22).

![Fig. 3](image-url) ITC profiles of poly (L-Glu)24 and poly (D-Glu)24 in H2O and D2O–H2O (4 : 1 v/v) at 30 °C.
d-glutamic acid in the turns which build the z-helix conformation, contributes approximately 0.1 kcal mol$^{-1}$ more than that of L-glutamic acid in their enantiomeric helical turns. Taking 3.6 as the number of residues in each turn of z-helix, the excess energy of $\sim 0.4$ kcal mol$^{-1}$ turn in poly(D-Glu)$_{24}$, is approximately equivalent to that of 10% of a hydrogen bond. In practice, ten z-helical turns of poly-d-glutamic acid would have an excess energy of an additional hydrogen bond compared to its poly-L-glutamic acid enantiomer. It is of great interest that in D$_2$O–H$_2$O 4 : 1, the above differences were found to induce a strong CD signal corresponding to a unidirectional helix formation. The energy difference between the left- and right-handed helices in these polymers is extremely small and the specific deuterium substitution sufficed for tipping the perfect balance between the enantiomeric helices. Analogously, the energy difference in enantiomeric polypeptides, like those used in this study, is undoubtedly very small. As stated above, even such small energy difference could be amplified to a detectable level. The mechanism we suggest for the chiral amplification process in polypeptides is presented below.

### General discussion

The application of polymers for the detection of chiral deviations has two main advantages. The first relates to the notorious effects of impurities in tested solutions, which in polymers is reduced to defects in the polymer matrix which can be eliminated by building it from highly purified monomers. The other advantage corresponds to intramolecular autocatalytic amplification of a slight chiral deviation which propagates along the polymer backbone. An example of such amplification was found in polysiloxanes, where a single stereospecific deuterium substitution in the side chains was found to induce a strong CD signal corresponding to a unidirectional helix formation. The energy difference between the left- and right-handed helices in these polymers is extremely small and the specific deuterium substitution sufficed for tipping the perfect balance between the enantiomeric helices. Analogously, the energy difference in enantiomeric polypeptides, like those used in this study, is undoubtedly very small. As stated above, even such small energy difference could be amplified to a detectable level. The mechanism we suggest for the chiral amplification process in polypeptides is presented below.

In the absence of an external discriminatory factor such as circularly polarized light, PVED remains the only physical effect which can lead to a selective and repetitive chiral enhancement. As PVED between isolated chiral isomers is extremely small, any expansion to the macroscopic realm must be associated with additional processes which lead to PVED amplification. In our poly-amino acid systems, amplification of PVED could, in principle, operate in two independent, yet cooperating, processes. The first is autocatalysis of helix formation or breaking, as clearly indicated in the ITC profiles of poly-amino acids. The other advantage corresponds to intramolecular autocatalytic amplification of a slight chiral deviation which propagates along the polymer backbone. An example of such amplification was found in polysiloxanes, where a single stereospecific deuterium substitution in the side chains was found to induce a strong CD signal corresponding to a unidirectional helix formation. The energy difference between the left- and right-handed helices in these polymers is extremely small and the specific deuterium substitution sufficed for tipping the perfect balance between the enantiomeric helices. Analogously, the energy difference in enantiomeric polypeptides, like those used in this study, is undoubtedly very small. As stated above, even such small energy difference could be amplified to a detectable level. The mechanism we suggest for the chiral amplification process in polypeptides is presented below.
The additional putative amplification arm is exclusive for aqueous solutions and corresponds to PVED induction of differences in the hydration layer between poly t-amino acids and their enantiomeric poly d-amino acids. In 2002 Tikhonov and Volkov reported an unexpected effect concerning proton exchange between the two spin isomers of H$_2$O, namely ortho-H$_2$O, where the proton spins are parallel, and para-H$_2$O, where the proton spins are anti-parallel. They have shown that the exchange rates between these isomers are much slower than expected (half life of 30–50 min.). Further evidence for this unexpected slow exchange has been recently reported.

One reasonable implication of this finding is that bulk water can be practically viewed as a mixture of ortho-H$_2$O and para-H$_2$O in a 3 : 1 ratio (due to the three degenerate states of ortho-H$_2$O). We have previously proposed that, since ortho-H$_2$O bears a magnetic field, it has a slight preference to react with t-enantiomers due to their PVED induced magnetic component. As a result, in aqueous solutions of racemic mixtures, solvation preference of t-enantiomers may take place, which in the extreme cases may lead to chiral enhancement and further to a selective separation. In line with this hypothesis, a polypeptide of t-amino acids in water might be solvated slightly more than its mirror image poly-d-amino acids, so that the latter adopts an apparently more hydrophobic nature. In poly(t-Glu)$_{24}$, poly(o-Glu)$_{24}$, poly(t-Lys)$_{24}$ and poly(o-Lys)$_{24}$, where the asymmetrical conformations are determined almost exclusively by solvation energy and intramolecular hydrogen bonding, the structures and their transition energies in water could thus become significantly different.

If this hypothesis is correct, then the spin isomers of H$_2$O and their putative selective effect on chiral isomers should be greatly diminished in D$_2$O or D$_2$O–H$_2$O mixtures. Indeed, this is what we have found. In our ITC experiments we have used a mixture of D$_2$O–H$_2$O 4 : 1 (v/v) as a system analogous to H$_2$O but with "scrambled" spin isomers, namely, largely devoid of enantiomeric preference. In the steady state, this mixture is constituted of 4% H$_2$O, 32% HDO and 64% D$_2$O and from all physical and chemical aspects resembles pure H$_2$O. As shown in Fig. 3, the pronounced differences in ITC profiles in H$_2$O between poly(o-Glu)$_{24}$ and poly(t-Glu)$_{24}$ are by and large abolished in 80% D$_2$O, as summarized in Table 1. Furthermore, the main effect of this mixture is on (L-Lys)$_{24}$ and poly(D-Lys)$_{24}$, where the asymmetrical conformations are determined almost exclusively by solvation energy and intramolecular interactions between residues in poly-o-glutamic acid may, by virtue of the relatively higher hydrophobicity of the o-isomer, be more pronounced. These will cause a higher restriction of the number of conformations, and create in the random coil a favorable backbone conformation of a higher tendency towards $\alpha$-helix formation in the poly-o-glutamic acid.

Results indicating chiral discrimination between t- and t-alanine at their crystalline or aggregated states have already been provided by Wang et al.

The results reported here imply that polypeptides comprised of t and l-amino acids may have slightly different structures and transition energies than their mirror image enantiomeric polypeptides. This difference could be reflected in a difference in catalytic activity between such pairs of polypeptides, which may provide a clue for the selection of l-amino acids in the origin of life. In future work, this possibility could be tested with enantiomeric peptides which bear a catalytic site. This may clarify the thermodynamic advantage in the presumed chiral selection proposed in this study.

Acknowledgements

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References

19. The optical purity examined by Nova Biochem was ≤0.5% while enantiomeric impurities were undetectable and were therefore assessed as <0.1%. The overall purity of the reacting compounds was assessed by HPLC to be higher than 99.5%.