TABLE I: Comparison of Calculated and Measured Diffraction Efficiencies in the Wavelength Range 633–752 nm for a BR<sub>DEN</sub> Film

<table>
<thead>
<tr>
<th>λ, nm</th>
<th>η&lt;sub&gt;calc&lt;/sub&gt;, %</th>
<th>η&lt;sub&gt;exp&lt;/sub&gt;, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>633</td>
<td>3.83 ± 0.57</td>
<td>3.40</td>
</tr>
<tr>
<td>647</td>
<td>6.06 ± 0.91</td>
<td>4.70</td>
</tr>
<tr>
<td>676</td>
<td>4.07 ± 0.94</td>
<td>3.51</td>
</tr>
<tr>
<td>752</td>
<td>1.07 ± 0.46</td>
<td>0.85</td>
</tr>
</tbody>
</table>

*IOD<sub>750</sub> = 5.0 and pH = 9.5 for a hologram recorded at an intensity of 5 mW/cm<sup>2</sup> per beam at 586 nm.

range. All experimental data are about 20% lower than those numerically derived, but the spectral dependence is correctly predicted and a maximal diffraction efficiency of 4.7% is found at 647 nm.

Summary and Conclusion

In this paper, the simultaneous measurement of the spectral dependence of the light-induced absorption and refractive index changes at high substrate conversion rates is described for BR films containing BR<sub>WT</sub> and BR<sub>DEN</sub>. The extended M lifetime of the BR<sub>DEN</sub> material is the main reason for the observed, approximately 4 times higher, amplitudes for the changes of both the absorption and the refractive index in the whole wavelength range of 400–800 nm. The spectral dependence of both values is identical for BR<sub>WT</sub> and BR<sub>DEN</sub>. The maximal refractive index change of 0.008 was observed for a BR<sub>DEN</sub> film of IOD<sub>750</sub> = 5.0, pH = 9.5, and a thickness of 25 μm at 633 nm at an intensity of 20 mW/cm<sup>2</sup> of actinic light of 568 nm. It was shown that the spectral dependence of the refractive index is in good agreement with the values derived from the spectral absorption changes by the Kramers–Kronig relation. This demonstrates that no major chromophore–matrix interactions occur which influence the refractive index change. The retinal-containing chromophore of BR in its amino acid cage can be treated in a first approximation as an almost undisturbed chromophoric group with respect to the photorefractive properties in the investigated intensity range. If the spectral dependence of the holographic diffraction efficiency η(λ, I) is in close agreement with the values calculated by Kogelnik’s formula. For the further characterization and optimization of BR films, a reliable prediction of η(λ, I) and a qualitatively correct estimation of the absolute diffraction efficiency can be derived from a difference spectrum and a single measured value of the refractive index change.

Acknowledgment. We thank D. Oesterhelt and Ch. Bräuchle for fruitful discussions. We acknowledge the financial support by the “Bundesministerium für Forschung und Technologie”.

References and Notes


Enthalpy of Knotted Polypeptides

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This paper concerns the estimation of the enthalpy of knotted conformers of poly-l-alanine, using a molecular mechanics force field. We have evaluated the relative energies of a variety of conformers of poly-l-alanine, including knots, for the size regimes of 58 and 124 residues. The knots investigated include right- and left-handed knots, knots containing helical secondary structure, supersecondary structure knots formed by turns in a helix to fold the helical backbone into a knot, and knots of varying degrees of tightness. While often the entropic barrier is cited for lack of observed knots in existing protein native structures, we find that the enthalpic barrier to knot formation is at least as formidable, if the ends of the carbonic anhydrase polypeptide chain were pulled in opposite directions, a knot would result. However, this is a marginal and exceptional case which in our view does not qualify as a genuine knotted structure.

Why are there no knotted proteins? One plausible reason is that a large entropic barrier makes it highly improbable for a knotted fold to occur. From this perspective it has been suggested that the observed (unknotted) native structure is a kinetically

Introduction

One striking observation concerning protein native conformations is that well-defined knotted structures do not occur in the current database of crystallized proteins. Our definition of a knot is the shoelace tie of the polypeptide backbone and not knots which result from threading through loops formed by covalent disulfide bonds. One protein, carbonic anhydrase, shows the last residue's carboxyl end just barely threading through a backbone loop region;
According to the above thermodynamic picture, the knotted structure would be kinetically stable minimum while a knotted structure is the thermodynamic minimum; the two minima are separated by an enormous free energy barrier which is largely entropic in origin. The enthalpic (energetic) calculations performed in this paper are consistent with the view that knots are energetically costly and that "native" states are actually lower in free energy.

We have used the parameters of the extended atom representation (version 19) of CHARMM. The first four terms refer to the potential functions with force constants \( k_r, k_\theta, k_\phi \). To our knowledge, no one has tried to quantify the energetic or enthalpic (or entropic) barrier to knot formation or has tried to assess the stability of knot conformers relative to other structural classes such as secondary, supersecondary, or tertiary structures.

We have investigated the question of knot stability by evaluating the relative potential energies for a variety of minimized conformers of a molecular mechanics representation of poly-L-alanine of 58-residue length: the supersecondary structure \( \alpha\text{-helix-turn-\alpha\text{-helix}} \) (Figure 2), the \( \alpha\text{-helix} \) (Figure 3), a globular "nativelike" structure very similar to BPT17*8 (Figure 4), a simple left-handed knot (Figure 5), a simple right-handed knot (Figure 7), and a simple left-handed knot with helical ends (Figure 8), and the highest energy conformer investigated in this work for length 58.

The procedure begins by choosing a harmonic penalty function on all heavy atoms and minimizing using the Powell procedure on the (L-alanine)_{58} hypersurface. After a rms derivative convergence of 0.1 kcal/(mol Å) is reached (or after the completion of 200 minimization steps), the penalty function force constant, \( k_p \), and the equilibrium value, \( r_0 \), are updated by reducing \( k_p \) by 5 kcal/(mol Å^2) and reassigning \( r_0 \) to be the position of \( i \) at the completion of the last minimization cycle. Once the energy penalty is totally eliminated, the structure is minimized using adopted basis Newton Raphson (ABNR) until the rms derivative is 0.005 kcal/(mol Å).

**Potential Energy Functions.** The empirical potential energy function used as the objective function, \( \Phi_o \), in this study has the form

\[
\Phi_o = \sum_{\text{bonds}} k_b(b_i - b_0)^2 + \sum_{\text{angles}} k_\theta(\theta_i - \theta_0)^2 + \sum_{\text{improper}} k_\gamma(\gamma_i - \gamma_0)^2 + \sum_{\text{improper}} k_{\gamma'}(1 + \cos(n\omega + \delta_i))^2 + \sum_{\text{torsions}} |C_4|q_i/r_{ij} + \epsilon_{ij}[(R_{ij}/r_0)^{12} - 2(R_{ij}/r_0)^6] \tag{1}
\]

In addition, the electrostatic interactions are scaled by a factor of \( C = 0.4 \) when the pair under consideration is separated by three covalent bonds. A cutoff of 7.5 Å is used for the evaluation of all pair interactions, using a shifting function to smooth the energy and derivatives. For further details of the specific CHARMM parameters, see ref 10.

We note that any empirical potential function contains inaccuracies; future improvements in protein force fields will warrant repeating some of the calculations presented in this work. However, we believe that any changes found would be of a minor quantitative character and that the main conclusions reached below concerning knot stability in the gas phase would survive. A potentially more limiting problem is the lack of explicit solvent effects in our calculations of knot stability. We simply state at the present time that this too will not change the qualitative nature of our conclusions, and we return to this point in the final section.

**Optimization Technique.** We have used a penalty function protocol for relaxing starting structures into a nearby local minimum. In each case, the starting structures were constructed by "hand". The procedure begins by placing a harmonic penalty function on all heavy atoms

\[
V_p = \sum_i k_p(r_i - r_0)^2 \tag{3}
\]

and minimizing using the Powell procedure on the (L-alanine)_{58} hypersurface. After a rms derivative convergence of 0.1 kcal/(mol Å) is reached (or after the completion of 200 minimization steps), the penalty function force constant, \( k_p \), and the equilibrium value, \( r_0 \), are updated by reducing \( k_p \) by 5 kcal/(mol Å^2) and reassigning \( r_0 \) to be the position of \( i \) at the completion of the last minimization cycle. Once the energy penalty is totally eliminated, the structure is minimized using adopted basis Newton Raphson (ABNR) until the rms derivative is 0.005 kcal/(mol Å).

**Results**

Table I displays the relative potential energies of the eight conformers of (L-alanine)_{58} in Figures 2–9. For the very few conformations we have considered besides the knotted forms, we have tried to take a representative from each of the following structural classes commonly observed in proteins: secondary, supersecondary, and tertiary. We have found that all three representative structural classes are much lower in energy than the knotted structures for (L-alanine)_{58}. The supersecondary structure \( \alpha\text{-helix-turn-\alpha\text{-helix}} \) is the most stable conformer (Figure 2). The \( \alpha\text{-helix} \) is the next
Figure 2. The supersecondary structure α-helix-turn-α-helix conformer of \((L\text{-ala})_{58}\). This is the lowest energy conformer investigated in this work for length 58.

Figure 3. The α-helix conformer of \((L\text{-ala})_{58}\). This is the second lowest energy conformer found in this study for residue length 58. For the 124-residue poly-L-alanine case, the α-helix is the lowest energy conformer.

Figure 4. A globular "native-like" structure very similar to BPTI. We have found this structure to be more stable than all knotted structures (Figures 5-8).

Figure 5. A simple right-handed knot. Although some favorable "packing" exists in the knotted form (relative to the extended conformer exhibited in Figure 9), this is largely offset by the loss of favorable steric and electrostatic interactions and geometric strain relative to the secondary, supersecondary, and tertiary structures displayed in Figures 2-4. The most stable conformer (Figure 3); apparently, the cost of the turn in the supersecondary structure is compensated by favorable nonbonded interactions between the two helical ends as they collapse against each other. The poly-L-alanine BPTI analogue (Figure 4) also has lower energy than any of the investigated knotted structures, although it is relatively high in energy compared to the ground state (~95 kcal/mol). Given these three representatives in structural class, we have considered the energy cost of knot formation for right- and left-handed knots containing no secondary structure (Figures 5 and 6) and with right- and left-handed knots with helical ends (Figures 7 and 8). Note that we have not used the inversion symmetry operation to obtain one handed conformer from another; in this case the energies would

Figure 6. A simple left-handed knot. A change in handedness offers no relief from the high-energy cost of knot formation.

Figure 7. A simple right-handed knot with helical ends. The folding of the loose end of the knot into helices provides a better comparison of the cost of knot formation as compared to the α-helix.

Figure 8. A simple left-handed knot with helical ends. See Figure 7 caption.

Figure 9. The extended energy conformer of \((L\text{-ala})_{58}\). This structure is higher in potential energy than the knotted conformers in Figures 5-8. This is due to the loss of nonbonded interactions whereby no part of the chain turns back onto itself to optimize "packing".
be the same since the two structures are mirror images of each other. Instead, we have built different starting conformers for the right- and left-handed conformers discussed below.

The simple knotted structures (right- and left-handed) with extended ends (Figures 5 and 6) are ~340 kcal/mol higher in energy than the supersecondary structure minimum. When the extended ends of the knotted structures are made helical (Figures 7 and 8), the energy cost diminishes to 172 (right-handed) and 258 kcal/mol (left-handed). In all cases of knotted structures, the energetic (enthalpic) barrier is formidable with respect to secondary, supersecondary, and even tertiary structure minima, due to a loss of stabilizing electrostatic and van der Waals interactions. For example, 60% of hydrogen bonds are lost when going from the fully helical structure (54 hydrogen bonds) to either simple knotted structure (23 and 18 hydrogen bonds for Figures 5 and 6, respectively). However, a comparison of the knots with helical ends (Figures 7 and 8) and the BFTI-like structure shows that hydrogen bond number is largely conserved (45 and 46, respectively), while neither structure is particularly well-packed.

Nonetheless, the more unfavorable electrostatic and van der Waals interactions of the knotted structures seem to indicate that these interactions cannot be optimized for the knotted forms. When the knotted conformer is compared to the energy minimum closest to the fully extended structure, one finds that knotting lowers the energy.

We have also investigated the dependence of knot stability on polypeptide length. Table II contains the relative energies for a variety of conformers of (l-ala)$_{14}$. The secondary and supersecondary structures are the lowest and next-lowest energy minima, respectively. However, the knotted structure with helical ends is more stable than the poly-l-alanine analogue of ribonuclease A, which is our chosen representative tertiary structure (a protein considered to contain large amounts of helix). This arises from the greater amount of helical secondary structure in the knotted conformer relative to the representative tertiary structure. The switchover in energy ordering of structural classes is a result of increase in polypeptide length. Knot formation in (l-ala)$_{10}$ is costly in energy relative to the tertiary structure with no knots, and little polypeptide is left for the energy compensation gained by helical ends. In contrast, (l-ala)$_{14}$ has ample polypeptide length to form relatively long, energy-stabilizing, helical ends after knot formation, in fact more helix than the tertiary structure analogue of ribonuclease A. Nonetheless, these simple knotted structures are still quite high in energy relative to the helical secondary and supersecondary conformers (~350 kcal/mol). However, again we observe energy stabilization of the knotted structure relative to the extended conformer, presumably due to favorable nonbonded interactions as a result of better packing.

The energy stabilization gained from the formation of helices and turns, and even knots when compared to the extended con-

### Table I: Relative Energies for a Variety of Conformers of (l-ala)$_{10}$

<table>
<thead>
<tr>
<th>Conformer</th>
<th>Energy (kcal/mol)</th>
<th>Conformer</th>
<th>Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-turn-α</td>
<td>0.00</td>
<td>α-knot(L)-α</td>
<td>257.89</td>
</tr>
<tr>
<td>α-helix</td>
<td>37.51</td>
<td>ext-knot(L)-ext</td>
<td>337.96</td>
</tr>
<tr>
<td>BFTI</td>
<td>94.59</td>
<td>ext-knot(R)-ext</td>
<td>346.87</td>
</tr>
<tr>
<td>α-knot(R)-α</td>
<td>172.18</td>
<td>extended</td>
<td>410.43</td>
</tr>
</tbody>
</table>

*Absolute energy ~1756.94 kcal/mol.

### Table II: Relative Energies for a Variety of Conformers of (l-ala)$_{14}$

<table>
<thead>
<tr>
<th>Conformer</th>
<th>Energy (kcal/mol)</th>
<th>Conformer</th>
<th>Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>0.00</td>
<td>α-knot-α</td>
<td>282.54</td>
</tr>
<tr>
<td>α-turn-α</td>
<td>24.22</td>
<td>ribonuclease A</td>
<td>356.93</td>
</tr>
<tr>
<td>tight helical knot</td>
<td>117.04</td>
<td>ext-knot-ext</td>
<td>734.16</td>
</tr>
<tr>
<td>loose helical knot</td>
<td>124.22</td>
<td>extended</td>
<td>875.86</td>
</tr>
</tbody>
</table>

*Absolute energy ~3697.10 kcal/mol.

The structure was chosen to optimize the amount of helical content, while at the same time forming a knot in the polypeptide chain. The energy cost is still quite high relative to the α-helix: ~125 kcal/mol.

Figure 10. A loose supersecondary structure knot conformer of (l-ala)$_{24}$. This structure was chosen to optimize the amount of helical content, while at the same time forming a knot in the polypeptide chain. The energy cost is still quite high relative to the α-helix: ~125 kcal/mol.

Figure 11. A tight supersecondary structure knot conformer of (l-ala)$_{24}$. Contrasting this structure with that in Figure 10, we find that the degree of tightness makes little difference in lowering the energy by optimizing "packing". The energy lowering relative to the structure in Figure 10 is only 7 kcal/mol.

The more unfavorable electrostatic and van der Waals interactions cannot be optimized for the knotted forms. When the knotted conformer is compared to the energy minimum closest to the fully extended structure, one finds that knotting lowers the energy.

We have investigated these conformers in order to see whether the favorable energetics gained by "packing" (i.e., comparing the energies of the more stable supersecondary structure α-helix-turn-α-helix with the α-helix of (l-ala)$_{14}$ and the energy stabilization of the knot relative to the extended conformer for both (l-ala)$_{10}$ and (l-ala)$_{14}$) overcomes the loss of favorable hydrogen-bonding interactions of a predominantly helix conformer. While these knotted structures are much lower in energy than the simple knots of (l-ala)$_{14}$ (~200 kcal/mol more stable), they are still ~115-125 kcal/mol higher in energy than the fully helical structure. Only a little stabilization occurs by making the knot tighter (~7 kcal/mol).

### Discussion and Conclusions

In conclusion, we have found that the thermodynamics of knot formation in poly-l-alanine is energetically very unfavorable relative to helical secondary and supersecondary conformers and less stable than some "globulelike" tertiary structures of small peptides (length less than 100 residues). In addition, combining the favorable features of helices and packing did not succeed in designing a stable knot relative to the helical secondary and supersecondary structures of (l-ala)$_{14}$. We therefore conclude that knot formation is energetically quite costly and that the energetic (enthalpic) contribution to the free energy barrier is at least as significant as the entropic component usually cited for the lack of knotted structures in proteins. Furthermore, the energetic instability is largely due to the loss of optimized nonbonded interactions, and only minor contributions from energy strain in the connectivity portion of the potential is observed. However, this loss of favorable nonbonded interactions cannot always be further characterized as nonconservation of hydrogen bond and/or van der Waals contact number, since we have found cases where knots are higher in energy even when hydrogen bonds and packing are roughly comparable between unknotted and knotted structures.

We emphasize again that this work only addresses the thermodynamic aspect of knot formation in polypeptides and only the enthalpic contribution at that. The kinetic question of quantifying the enthalpic barrier to knot formation in the initial stages of
folding is an important question as well. Whether an unfavorable enthalpic contribution to the free energy barrier is the same magnitude as the (unfavorable) entropy in the early kinetics will be a strong function of sequence; in fact, native sequences may actually bias the folding against sampling knotted conformations. Thus, the use of alanine homopolymer is an inadequate model for further addressing the early kinetics of knot formation.

The size regime at which we have looked corresponds to typical polypeptide lengths of real protein systems. Scaling theories indicate that knotting certainly is plausible for very long flexible chains under the right solvent conditions;\(^1\) random self-avoiding walks on a simple cubic three-dimensional lattice have also demonstrated that self-knotting is highly probable (tending to unity) as the polypeptide length increases. However, the lengths required for knot formation in these theories are extremely large for single-chain polymers and beyond typical protein lengths.

One shortcoming of the present study is the lack of sampling of knotted poly-L-alanine conformers. This is the usual problem faced when trying to characterize a region of a complex hypersurface in which the multiple minima problem is fierce and is further exacerbated by consideration of an especially odd region for which the empirical potential functions were not designed. However, if we are ultimately to address questions such as whether a native protein structure is a metastable or thermodynamic minimum, and how proteins in the size regime of 50–400 amino acids prevent self-knotting to reach known unknotted forms, we must begin to probe these regions.

We believe that the conclusions reached for the high (thermodynamic) energy cost of knots would not be severely altered by inclusion of aqueous solvent. The helix and knotted structures should both be energy destabilized in solvent relative to their gas-phase minimum. While the helix may be more destabilized relative to its gas-phase counterpart than the knotted structure, it is unlikely that the 115–350 kcal/mol barrier between helix and knotted conformers will be eliminated. Secondly, we have used the L-alanine homopolymer to demonstrate the existence of energy minima corresponding to knotted conformers, the use of a more realistic heteropolymer we believe will only exacerbate the already high thermodynamic energy cost of knot formation, in that bulky and/or charged side chains will find the knotted conformer too crowded for favorable steric and electrostatic interactions. This is a result of alanine's small, uncharged side chain, which gives it a larger degree of structural plasticity than all other side chains except glycine.\(^8\) Finally, while we believe that native sequences will not exhibit low-energy knotted structures, a sequence marching to the beat of its own drummer, i.e., a synthetically designed knot in the form of judiciously placed glycines and prolines for example, might well exhibit the remarkable thermodynamic stability that native sequences are unable to obtain.

Registry No. L-alanine (homopolymer), 25191-17-7; poly-L-alanine (SRU), 25213-34-7.

References and Notes


\(^{23}\text{Na NMR of Concentrated DNA Solutions: Salt Concentration and Temperature Effects}^{23}\)

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Aqueous solutions of short DNA fragments (147 base pairs) with DNA concentrations ranging from 10 to 257 mg/mL at three supporting electrolyte concentrations (0.01, 0.1, and 1.0 M Na\(^+\)) were examined by \(^{23}\text{Na NMR}\) at temperatures from 20 to 60 °C. The longitudinal relaxation rate (T\(_1\)) decreased with increasing temperature at all DNA and added salt concentrations, indicating that the exchange between free and bound counterions was fast on the NMR time scale. T\(_1\) increased linearly with increasing DNA phosphate/Na ions ratio until the onset of anisotropic phase formation, where the increase was nonlinear. Analysis of this behavior in terms of a two-state model of counterion binding is consistent with changes in both the fraction of bound ions and their relaxation rate upon anisotropic phase formation. Spectra of anisotropic samples at all three salt concentrations exhibited quadrupole splittings correlated with the appearance of the cholesteric phase. The magnitude of quadrupole splitting never exceeded 400 Hz and changed in a complex way with DNA concentration and temperature. The experimental results are analyzed in terms of existing theoretical models of DNA–counterion interactions in dilute and concentrated DNA solutions.

Introduction

DNA packaging in vivo is highly efficient and DNA concentrations in phage heads and eucaryotic nuclei are of the order of hundreds of milligrams per milliliter of volume. In some cases this "high-density state" corresponds to a spatially ordered arrangement of DNA molecules, usually referred to as a "liquid-crystalline" state. Sipkis and Wagner showed that a large, negative ellipticity observed in the CD spectra of chromosomal preparations from equine sperm cells can be explained if a cholesteric arrangement of DNA molecules in the chromosomes is assumed. Liquid-crystalline organization of DNA molecules was also proposed for diniflagellate (Prorocentrum micans) chromo-