

# Protein plasticity to the extreme: changing the topology of a 4- $\alpha$ -helical bundle with a single amino acid substitution

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**Background:** Conventional wisdom has it that two proteins sharing 98.4% sequence identity have nearly identical three-dimensional structures. Here we provide a counter-example to this statement by showing that a single amino acid substitution can change the topology of a homodimeric 4- $\alpha$ -helical bundle protein.

**Results:** We have determined the high-resolution crystal structure of a 4- $\alpha$ -helical protein with a single alanine to proline mutation in the turn region, and show that this single amino acid substitution leads to a complete reorganisation of the whole molecule. The protein is converted from the canonical left-handed all-antiparallel form, to a right-handed mixed parallel and antiparallel bundle, which to the best of our knowledge and belief represents a novel topological motif for this class of proteins.

**Conclusions:** The results suggest a possible new mechanism for the creation and evolution of topological motifs, show the importance of loop regions in determining the allowable folding pathways, and illustrate the malleability of protein structures.

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## Introduction

The Rop (repressor of primer) protein is a homodimeric RNA-binding protein involved in the regulation of the copy number of the ColE1 plasmid [1]. Rop is the paradigm of a canonical 4- $\alpha$ -helical bundle [2] and, as such, has been the subject of numerous investigations ranging in their approach from structural [3–6] and biochemical [7,8], to thermodynamical [9–11] and computational [12] studies. The bend region of Rop has attracted added interest, not least because of the ongoing debate about the role of loops in the folding and stability of bundles and proteins in general [9,13–18]. Although random mutagenesis experiments suggested that most single amino acid substitutions in the bend region of Rop can be tolerated by the native structure [16], one of the designed site-directed mutants — the Ala31→Pro (A31P) mutant — showed consistent and persistent deviations from the thermodynamical and biochemical properties of the wild-type protein [7,16,18]. The crucial role of Ala31 in the formation of the turn region of wild-type Rop was first recognised in the original structure determination of the protein [3]. This residue not only has unusual geometry — as judged from its  $\phi, \psi$  angles, and the exceptional deviation of its peptide unit from planarity ( $\omega = 194.3^\circ$ ) — but is also unique in being the only amino acid that simultaneously forms hydrogen bonds to both helices [3,18].

We designed the A31P mutant in the hope that the conformationally constrained proline could not mimic alanine in its role as the central residue of the turn, and would thus result in a structure with a partly unfolded turn. This would allow us to tackle a long standing, and controversial, issue regarding the relative contribution of turn–helix interactions to the overall stability of the bundle [9,13,15,16].

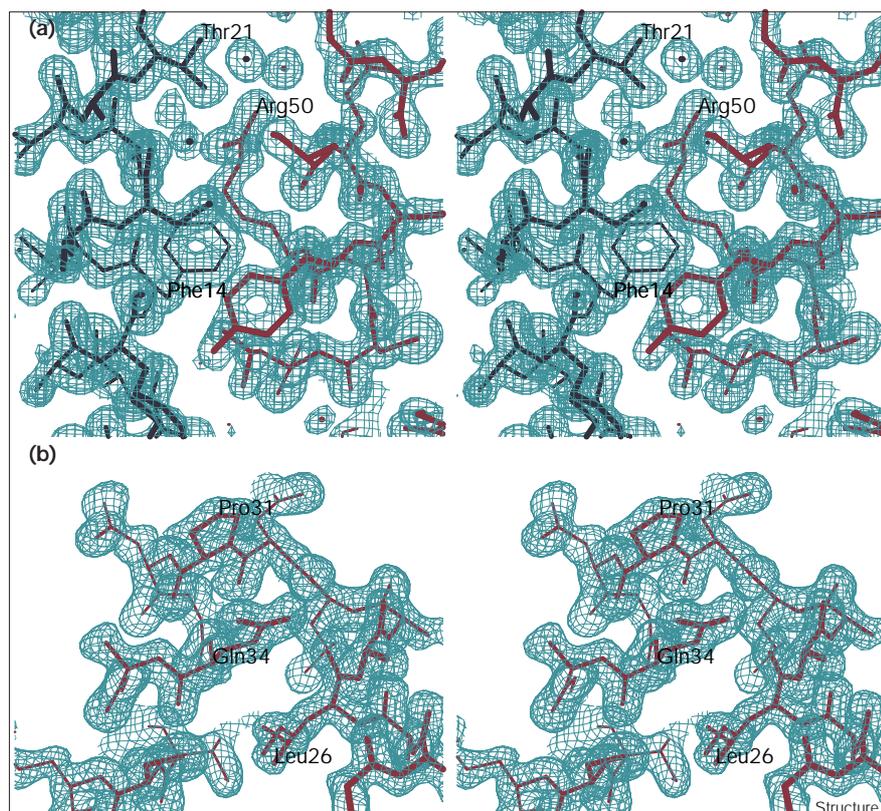
Instead of providing an answer to this problem, the crystal structure of the A31P mutant answered the fundamentally different, and as yet unasked, question of how many mutations are needed to change the topology of a protein (which, as it happens, is a generalisation of the ‘Paracelsus challenge’ [19,20]). In the following paragraphs we compare the topologies and tertiary structures of the wild-type and mutant Rop proteins, describe in some detail the structure of the turn and the mode of hydrophobic core packing, and discuss the implications of the A31P crystal structure with respect to the thermodynamical [18] and functional [7] data available for this mutant.

## Results

### Topological changes

A summary of the structure determination procedure for the Rop A31P mutant is given in the Materials and methods section. The electron-density maps in Figure 1

Figure 1



Stereoview diagrams of the electron-density distribution with the final model superimposed. (a) The map shows a large volume around a crystal-packing contact between the first (black) and second (red) helices of the monomer. Residues Phe14, Thr21 and Arg50 have been labelled. The map is contoured at  $1.5\sigma$  above the mean, and all density features in the volume shown are drawn. (b) The distribution of density around the turn region. Residues Pro31, Gln34 and the discretely disordered Leu26 are labelled. For clarity, contours have only been drawn around the protein model. The electron density in both (a) and (b) corresponds to a sharpened  $\sigma_A$ -weighted difference map [28] of the form  $(2mF_o - DF_c)^{0.6} E_{2mF_o - DF_c}^{0.4} \exp(i\phi_c)$ , where  $E_{2mF_o - DF_c}$  are the normalised structure-factor amplitudes corresponding to  $2mF_o - DF_c$ . All data between infinity and 1.8 Å have been used for the calculation. (The plots were prepared using the programs O and Oplot [30].)

illustrate the quality of phase determination and show a detailed view of the electron-density distribution around the turn region and the mutated proline residue. In Figure 2, the topological diagrams and overall structures of the wild-type and mutant proteins are compared. Although both proteins are homodimeric and each is composed of two identical (crystallographically related) monomers, different names are used for the individual helices in order to simplify the comparison (see Figure 2 for the naming convention for the helices of the two structures). Topologically speaking, the operation that converts the wild-type protein to the mutant structure, and vice versa, is one of a mutual exchange of position (but not connectivity or polarity) of two neighbouring helices that belong to different monomers (i.e. the pairs  $A \leftrightarrow C$  or  $B \leftrightarrow D$  in Figure 2) while keeping the other two helices unchanged. This operation has two consequences. The first consequence is that the handedness of Rop is inverted, and is transformed from a left-handed to a right-handed bundle [21]. The second consequence is that any one of the four helices is now surrounded by one parallel and one antiparallel helix (parallel pairs A–D and B–C; antiparallel pairs A–C and B–D).

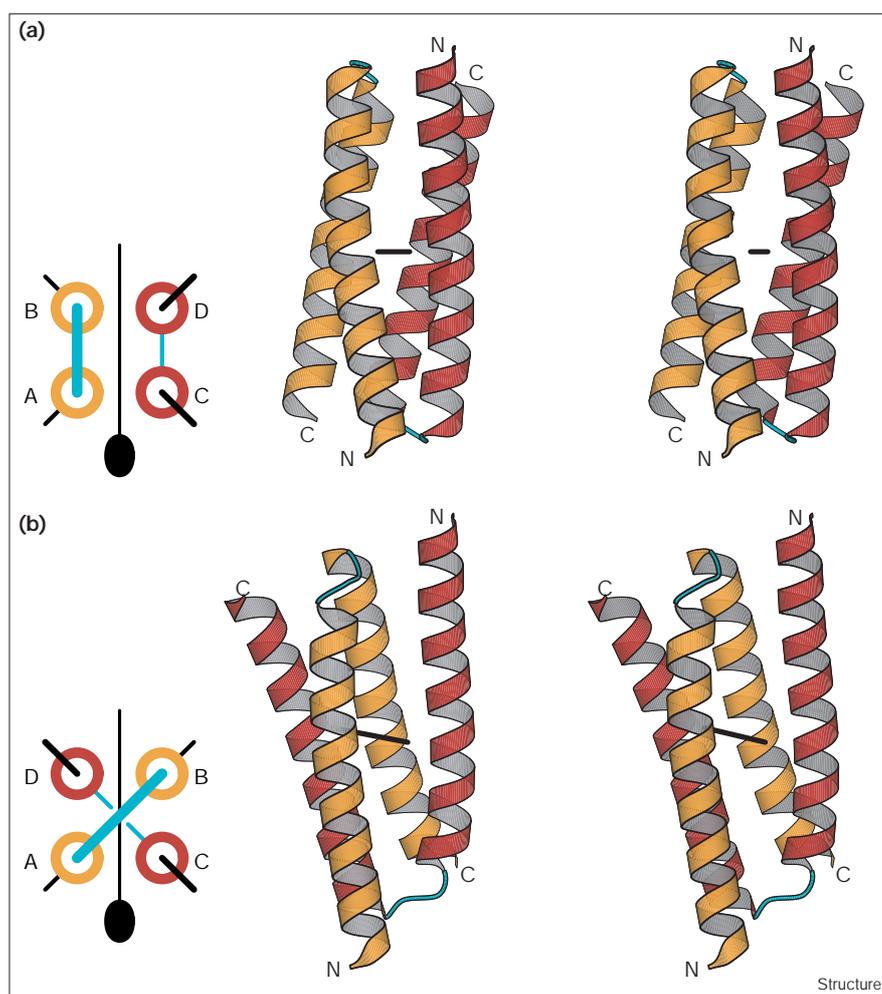
#### Changes in the tertiary structure

The abstract simplicity of the topological diagrams in Figure 2, and of the corresponding transformation relating

them, hides the fact that the helices have an asymmetric disposition of sidechain groups, which evolved under the restraints of a left-handed, all-antiparallel bundle: changing one residue in the turn region can change the relative position and orientation of the helices, but does not change the distribution of hydrophobic, polar or charged sidechains on their surfaces. In view of this, it is not surprising that the comparison between the overall structures (shown in Figure 2) has lost something of the simplicity implied by the topological transformation presented above. The most obvious difference is the increase of the interhelix angle of each monomer by  $\sim 16^\circ$  in order to accommodate the turn of the other monomer. This widening of the interhelix angle is the result of a much more complex movement. If the wild-type and mutant monomer structures are superimposed using only the A (or C) helices, and the new position and orientation of the corresponding B (or D) helices is compared, we find that these helices are related by a series of transformations: a rotation of  $118^\circ$  about a helix axis, followed by a rotation of  $37^\circ$  about an axis perpendicular to the plane containing the helical axes and a translation of 14.3 Å (these changes can be seen in Figure 2 by comparing the position and orientation of the D helices from the wild-type and mutant structures). These transformations result in a mean separation between the equivalent C $\alpha$  atoms of the least-squares superimposed wild-type and A31P monomers

Figure 2

Comparative schematic diagrams of the topology and overall structure (in stereo) of (a) wild-type Rop and (b) the A31P mutant. The helices of each monomer are shown in red and yellow and the connective strands are in blue. The line thickness in the topological diagrams is inversely proportional to the distance from the viewer. The position of the intramolecular (crystallographic) dyad axis is noted both in the topological diagrams and the structure schematics. The orientations of the wild-type and mutant structures are such that the N-terminal helices of the red monomers (C helices) are in exactly the same position and orientation in both diagrams. The N and C termini as well as the helix naming convention adopted are also noted. (The structure schematics were prepared using the program BOBSCRIPT [31].)



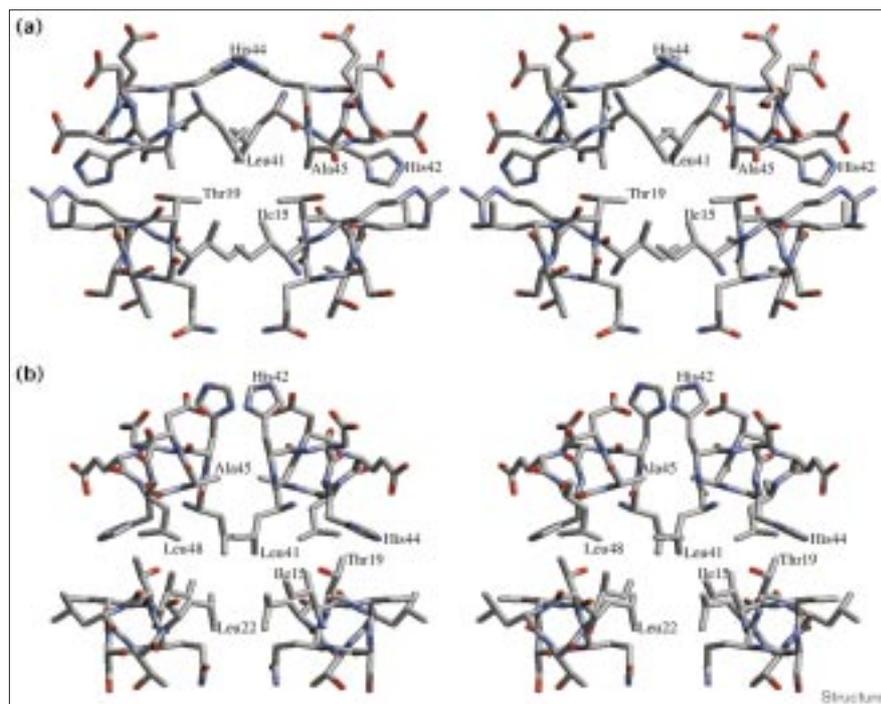
(residues 1–56) of  $\Delta = 4.55 \text{ \AA}$ , with a standard deviation ( $\sigma_{\Delta}$ ) of  $5.05 \text{ \AA}$  and a maximum separation ( $\max_{\Delta}$ ) of  $9.66 \text{ \AA}$  (as calculated using the program LSQKAB [22]). The second important difference between the overall structures of the two proteins is that A31P is no longer a left-handed four-stranded coiled coil. The curvature and relative orientation of the pairs of helices C–B and A–D would suggest that A31P might be a left-handed coil of two right-handed two-stranded coiled coils. However, examination of the mean helix–helix distances (D–B  $9.5 \text{ \AA}$ , A–C  $13.5 \text{ \AA}$ , A–D and C–B  $10.6 \text{ \AA}$ ), of the orientation angles [23] (D–B  $-169^{\circ}$ , A–C  $-173^{\circ}$ , C–B and A–D  $-8^{\circ}$ ; diagonal pairs C–D and A–B  $+175^{\circ}$ ) and of the packing interactions between adjacent helices, suggests that only helices D and B might indeed form a two-stranded coiled coil with a left-handed twist. For the other pairs of helices no conclusions can safely be drawn.

#### The bend and the helices

Turning our attention to the bend region and the individual helices, we note that the length and location of the

turn has changed: in wild-type Rop the bend comprises three residues (Leu29, Asp30 and Ala31), whereas in the A31P structure the turn starts at Asn27 and finishes with Asp30. As shown in Figure 1b, the mutated proline is in the *trans* isomer and is not directly involved in the turn formation. Instead, it has the role of N-cap for the second helix of the monomer (helices B and D; Figure 2). Two major geometric changes are responsible for the different relative position and orientation of the helices of each monomer in the mutant and wild-type structures: a change of the  $\psi$  angle of Leu29 by  $169^{\circ}$ ; and a change of the  $\phi$  and  $\psi$  angles of Asp30 by  $131^{\circ}$  and  $119^{\circ}$ , respectively. Although a comparison between the structures of the individual helices (helix A or B from wild type with helix A or B from A31P) shows less impressive differences ( $\Delta = 0.70 \text{ \AA}$ ,  $\sigma_{\Delta} = 1.16 \text{ \AA}$ ,  $\max_{\Delta} = 5.28 \text{ \AA}$  for all equivalent atoms of residues 1–26;  $\Delta = 1.57 \text{ \AA}$ ,  $\sigma_{\Delta} = 2.16 \text{ \AA}$ ,  $\max_{\Delta} = 9.85 \text{ \AA}$  for all equivalent atoms of residues 32–56) it is still somewhat surprising to observe a standard deviation in the atomic positions of the order of  $2 \text{ \AA}$  for two

Figure 3



Comparative all-atom stereoview diagrams of a central slice from (a) the wild-type Rop and (b) the A31P mutant structures. The intramolecular dyad axis is parallel to the plane of the paper and the structures are oriented as shown in the topological diagrams of Figure 2. The residues forming the first hydrophobic layer in the two structures are labelled, as are His42 and His44. In wild-type Rop, residues Leu22 and Leu48 belong to the second hydrophobic layer and for clarity have not been drawn. For residues with alternative sidechain conformations (see text), only the major conformer is depicted. (The figure was prepared using the program RASMOL [22].)

identical sequences with the same secondary structure. This, we believe, shows how the detailed atomic arrangement of even a relatively rigid structural element, such as an  $\alpha$  helix, is influenced — if not determined — by its environment. (It is worth noting that the observed differences do not arise solely from the sidechain atoms. When only the mainchain atoms are used for the least-squares superposition, we still observe a  $\max_{\Delta} = 1.88 \text{ \AA}$  for the first helix and a  $\max_{\Delta} = 3.37 \text{ \AA}$  for the second).

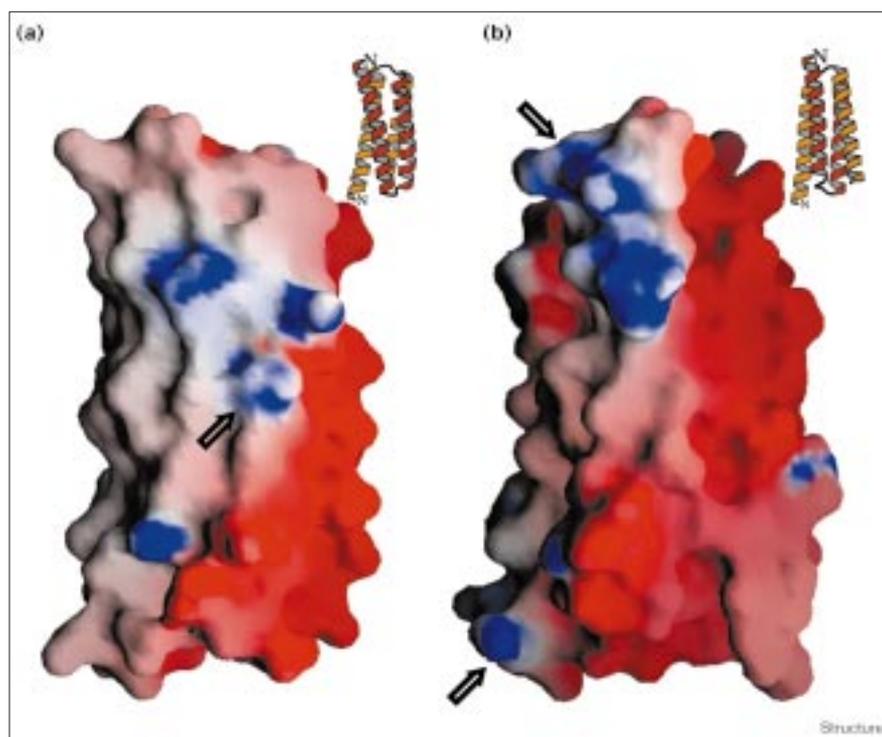
#### A new hydrophobic core

In view of all these structural changes, it comes as no surprise that the hydrophobic core of the A31P mutant is packed anew. The hydrophobic interactions in the central part of the wild-type and mutant structures are compared in Figure 3. It is immediately obvious from this comparison that at the atomic level the two proteins diverge so much that it makes more sense to note similarities instead of differences. In this spirit, we note that remnants of the layered structure of the hydrophobic core, as described for the wild-type protein [3], are still present in the mutant structure. In the case of A31P, however, there are only six (instead of eight) such layers and of these the two outer ones (closest to the turns) consist of clusters of only three amino acids each, and can probably be called layers only for reasons of consistency with the native protein. Furthermore, the planes of these layers are highly tilted with respect to the bundle axis (instead of being approximately normal to it, as is the case for the wild-type protein) and

their tilt angle increases proportionally to their separation from the intramolecular dyad axis. In the case of the wild-type protein, these hydrophobic layers consist exclusively of amino acids that occupy specific (and constant) positions with respect to the heptad sequence periodicity characterising associating  $\alpha$  helices, and they all have the form *adad*, where *a* and *d* are the generally apolar positions of the repeat [2,3] (the other five positions of the heptad repeat *b*, *c*, *e*, *f* and *g* are generally occupied by polar residues). Although A31P shares exactly the same periodicity — or lack of it in the turn region — the distribution of amino acid types in its layers is totally different. For example, the two central layers closest to, and related by, the dyad axis (shown in Figure 3) have — in accordance with the wild-type heptad assignments — the form *dddd*. The next two layers are even more divergent in composition: each consists of five residues (Met11, Ile37, Cys38, Cys52 and Leu26) and have the form *ggaaa*. Finally, the two outermost layers comprise Ile37 (only the C $\delta$  atoms, see below), Leu29 and Ala8 and have the form *gdd*. Two other features of the A31P structure are worth noting. The first is the presence of a large continuous internal cavity (with a volume [24] of  $\sim 270 \text{ \AA}^3$  for a probe with a radius of  $1.4 \text{ \AA}$ ), which is located around the dyad axis and is surrounded by the first pair of hydrophobic layers (the presence of the cavity can also be inferred from Figure 3b). The second feature is the large number (five per monomer) of internal hydrophobic sidechains for which the electron-density maps suggested the presence of at

Figure 4

Comparisons of electrostatic surface potential. The molecular surface of (a) wild-type Rop and (b) the A31P mutant colour-coded according to the electrostatic potential from  $-10$  kT/e (red) to  $+10$  kT/e (blue). The ribbon diagrams at the upper right-hand corner of each panel depict the orientation of the corresponding structures. Arrows point to differences in positive charges arising from the incomplete atomic modelling (due to the lack of convincing density) for the sidechains of Arg16 (A31P structure), Lys3 (native Rop) and Lys6 (native Rop). (The figure was prepared using the programs GRASP [24] and BOBSCRIPT [31].)



least one alternative conformation. Of these five sidechains, three belong to the first hydrophobic layer and their mobility can be attributed to the fact that they are in direct contact with the cavity mentioned above. Of the remaining two, one is the sidechain of Ile37, which bridges and participates in both the second and third hydrophobic layers and its disorder may be structurally significant, and the second is Leu26, which directly contacts Ile37 in the second hydrophobic layer.

#### Thermodynamic data

The crystal structure of A31P agrees with and qualitatively explains the available thermodynamic and spectroscopic data available for this mutant [18]. The observed destabilisation of the mutant ( $\Delta\Delta G = 29$  kJ/mole of dimer at  $25^\circ\text{C}$ ) is explained quite adequately in terms of the reduced number and density of the hydrophobic core packing interactions. The reduction of the helical content calculated from the crystal structures (4%) agrees quite well with the value (7%) obtained from circular dichroism measurements. The interpretation — in terms of a diminished interhelical interaction — of the reduced ratio of the ellipticity values  $[\Theta_{222\text{nm}}]/[\Theta_{208\text{nm}}]$  and of the reduced transition enthalpy of the mutant [18], is in very good agreement with the increased mean helix-helix distances. The conclusion that the observed thermodynamic and spectroscopic changes “... cannot be rationalized by the assumption of mere localized perturbations” [18] is fully supported by our results.

#### Functional data

It is more difficult to reconcile the crystal structure presented above with the finding that A31P retained some of the biological activity of the wild-type protein [7]. Actually, this mutant appears to be approximately as efficient in regulating the copy number of the ColE1 plasmid as a double alanine insertion mutant, the structure of which only shows minor and localised changes in the bend region [5]. We believe that at least part of the answer to this problem lies in the previously suggested [8] functional significance of the asymmetric disposition of charged sidechain groups on the surface of the Rop dimer. Figure 4 shows a comparison between the distribution of electrostatic potential on the molecular surface of the native and mutant structures [24]. It is obvious that the pronounced asymmetry in the distribution of the neutral and positively charged sidechains (facing towards the left-hand side in Figure 4) and of the negatively charged ones (towards the right-hand side) holds well, even for the mutant structure. The preservation of this asymmetry is due to the fact that the relative orientations of the symmetry-related helices A and C, which form the assumed RNA-binding site of Rop [7,8], (Figure 2) are not altered in the mutant structure. This is not to imply that the topology of the RNA-binding surface has remained unchanged: if we superimpose only helices A (or C) from the wild-type and mutant structures, and then compare the position and orientation of the corresponding C (or A)

helices, we find that they are related by a rotation of  $\sim 35^\circ$  followed by a translation of 4.8 Å (these changes can also be inferred from Figure 2). Even if the relative helix rotation is ignored, we find that the distance, for example, between the symmetry-related C $\beta$  atoms of Phe14, a residue known to be essential for RNA-binding [7,8], increases from 10.8 Å in wild type to 14.3 Å in the A31P mutant. Given that four different Phe14 mutants (phenylalanine to alanine, leucine, tyrosine and tryptophan) all failed to bind the wild-type RNA substrate [8], it is not clear how A31P, which displays such drastic structural changes, can still bind to it.

## Discussion

In summary, we have shown that a single amino acid substitution is sufficient to change the topology of a small protein, leading to drastic changes both in its surface properties and the packing of its hydrophobic core. Although the stability of the resulting structure is significantly reduced (compared with the wild type), the amount of structural differences observed between two proteins sharing 98.4% sequence identity, justifies our proposition that “the remarkable thing is that it fold at all” [20].

The A31P crystal structure supports the view that turns are not passive with respect to protein folding. Our results — and taking into account the experimentally demonstrated insensitivity of this same protein to numerous other mutations in the same turn region [16] — would suggest that the role of turns in protein folding is not one of actively determining the fold, but one of actively excluding some of the otherwise possible folding pathways. Clearly, when it is the major folding pathway that is being excluded, then whether or not the protein will fold will depend on the existence (or otherwise) of another permissible folding pathway leading to a stable molecule. This argument is valid even when inverted. It seems reasonable to suggest, for example, that the structure exhibited by the A31P mutant is also accessible by the native protein — after all, the difference in terms of composition between the two structures is only two atoms (the C $\gamma$  and C $\delta$  atoms of proline) and their bonds. If, in a thought experiment, these two atoms were removed from the A31P structure, we would end up with a perfectly normal (with respect to its  $\phi, \psi$  angles) alanine residue, and would have thus returned to the wild-type Rop sequence, but folded as observed in the A31P crystal structure. One of the reasons that the native molecule is not trapped in an A31P-like intermediate is because Ala31, but not Pro31, is permissible to a folding pathway that leads to a thermodynamically more stable conformation.

It would appear at first sight that our results place doubts on one of the basic premises of modern molecular biology, that is, the sequence/structure/function equivalence. We do not think that this is the case: the creation of a new

topology places new restraints on the protein sequence, and these new restraints will eventually lead to sequence divergence. Given enough time (and evolutionary events), the sequence of A31P, for example, would change to reflect the structural (and possibly functional) restraints of its new topology (i.e., a right-handed, mixed parallel and antiparallel 4- $\alpha$ -helical bundle). As a consequence, the protein would probably end up with a sequence having as much homology to the native Rop as we would presently expect from two structurally similar, but different in detail, proteins.

Although tempting to suggest, we do not believe that this abrupt way of generating a new topology corresponds to an evolutionarily frequent event. Not only does our general experience from mutagenesis studies shows that what we observed is a rare occurrence, but it is also hard to imagine such drastic changes happening to a larger, more complex and/or non-multimeric protein. We do suggest, however, that such events may have been of some importance in the early stages of molecular evolution.

## Biological implications

**The general experience from structural studies of mutant proteins with single amino acid substitutions is that the effect of mutation is rather localised and minor. We report here an exception to this rule by showing that a single alanine to proline substitution is sufficient for changing the topology of a small protein. The mutation leads to drastic changes both in the surface properties of the protein and the packing of its hydrophobic core, while retaining some of the biological activity of the wild-type molecule. The results exemplify the complexities of the folding problem and show that the sequence/structure/function equivalence should be treated with some caution in the case of non-natural products. In addition, our observations suggest a possible new mechanism for the creation and evolution of protein topologies, and underline the importance of loop regions in determining the allowable folding pathways.**

## Materials and methods

The expression, purification, crystallisation and preliminary crystallographic characterisation of A31P has been reported previously [7,25]. All crystallographic calculations were performed with the CCP4 suite of programs [22] and X-PLOR [26]. In summary, a 3.8 Å solvent-flattened single isomorphous replacement (SIR) map (SOLOMON [22], 30% solvent content), based on a single-site platinum derivative showed the approximate location of the helices and suggested that these may be parallel, but was otherwise uninterpretable. The structure was solved with a novel procedure (a detailed account of which will be published elsewhere) involving rigid-body simulated annealing (in X-PLOR) of roughly positioned polyaniline models at a very high initial temperature ( $T_0 = 10,000\text{K}$ ) and with the geometric energy terms switched on. The annealing procedure was iteratively repeated with successively smaller rigid bodies (down to two alanine residues per body), at successively higher resolution (5, 4, 3 and 2 Å) and converged to R and  $R_{\text{free}}$  values [27] of 0.403 and 0.413. These values were obtained for all data between 8 Å and 1.8 Å from a 100% complete native data set collected

on a CAD4 diffractometer (average  $F/\sigma(F) = 12.4$  for all data, 2.51 for the last resolution shell). A  $\sigma_A$ -weighted map [28] of the form  $(2mF_o - DF_{Ala52}) \exp(i\phi_{Ala52})$  was readily interpretable in terms of the protein sequence, and was further improved with the wARP procedure [29]. Sidechains were built using the program O [30] and the refinement was completed with rounds of model building in O and conjugate gradient refinement in X-PLOR. The final model comprises 56 residues (Met1–Phe56) and 55 water molecules, with the terminal atoms of the solvent-exposed sidechains of Leu9, Arg16, Asn27 and Arg55 excluded due to lack of convincing density. This model has an R factor of 0.188 and an  $R_{free}$  of 0.240 for all data between infinity and 1.8 Å ( $R = 0.149$  and  $R_{free} = 0.191$  for all data with  $F/\sigma(F) > 3.0$ ). The model scores better than average on all of the PROCHECK [22] tests, giving an overall G factor of +0.56 with 100% of the residues in the core Ramachandran regions. The average standard deviation for mainchain bond lengths and angles is 0.007 Å and 0.909°, respectively, and the B factor root mean square deviation for mainchain and sidechain bonds is 1.55 Å<sup>2</sup> and 2.77 Å<sup>2</sup>, respectively.

#### Accession numbers

The atomic coordinates for the A31P mutant have been deposited with the Protein Data Bank (accession code 1b6q).

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