

## $D_n$ -Symmetrical Tertiary Templates for the Design of Tubular Proteins

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Antiparallel helical bundles are found in a wide range of proteins. Often, four-helical bundles form tube-like structures, with binding sites for substrates or cofactors near their centers. For example, a transmembrane four-helical bundle in cytochrome  $bc_1$  binds a pair of porphyrins in an elongated central cavity running down the center of the structure. Antiparallel helical barrels with larger diameters are found in the crystal structures of TolC and DSD, which form antiparallel 12-helical and six-helical bundles, respectively. The backbone geometries of the helical bundles of cytochrome  $bc_1$ , TolC, and DSD are well described using a simple  $D_n$ -symmetrical model with only eight adjustable parameters. This parameterization provides an excellent starting point for construction of minimal models of these proteins as well as the *de novo* design of proteins with novel functions.

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**Keywords:** *de novo* protein design; coiled coil;  $\alpha$ -helical barrel; TolC, cytochrome  $bc_1$ ; domain-swapped dimer

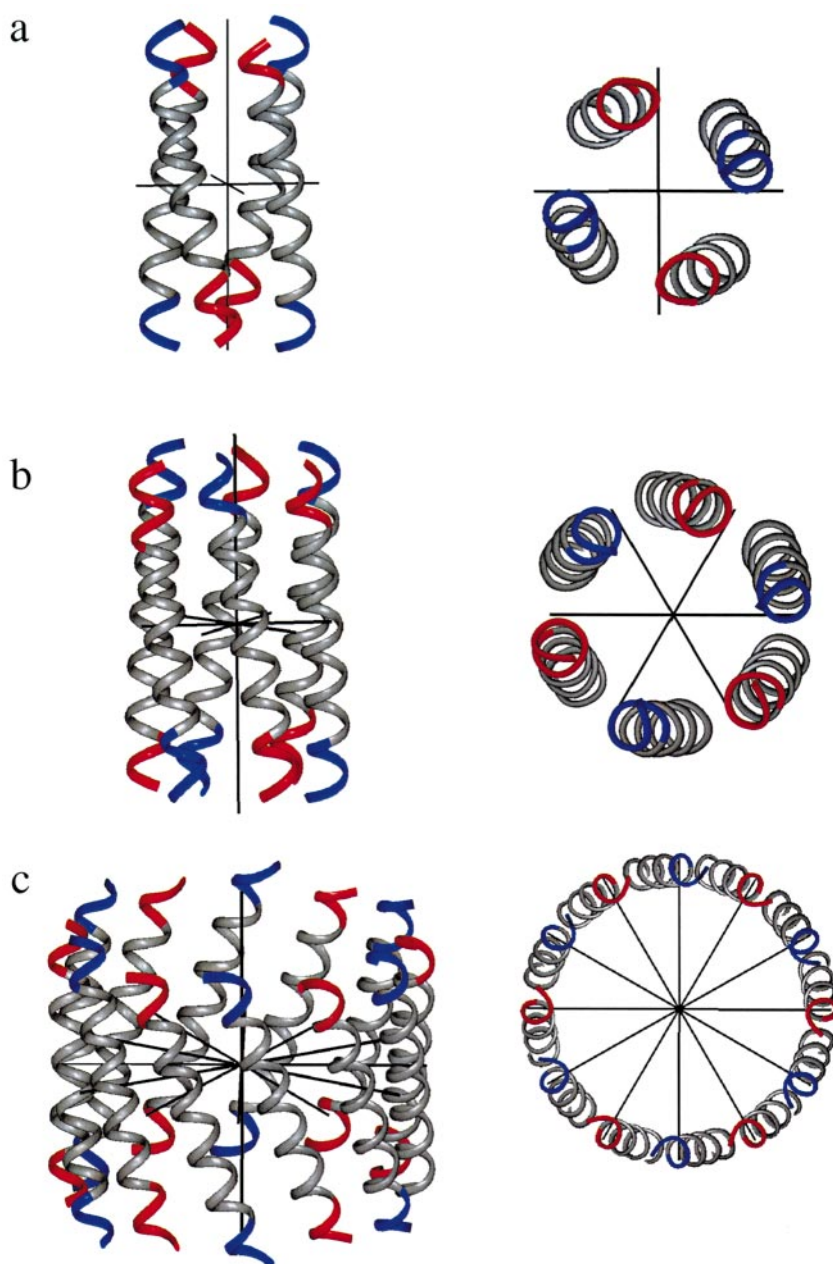
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### Introduction

*De novo* protein design provides an attractive approach to probe the features required for the folding and function of proteins. Recently, much work in this area has focussed on the design of parallel coiled coils,<sup>1,2</sup> which comprise a simple oligomeric folding motif that is frequently found in fibrous proteins as well as the oligomerization domains of globular structures. Parallel coiled coils have approximate or exact  $C_n$  symmetry, in which  $n$  parallel helices are arranged about a central superhelical axis. A number of  $C_n$ -symmetrical coiled coils have been designed and characterized crystallographically, including dimeric, trimeric, and tetrameric bundles of helices.<sup>3–8</sup> Antiparallel helical bundles represent a second class of folding motifs, which are functionally more diverse than parallel coiled coils. For example, antiparallel three-helix<sup>9</sup> and four-helical bundles<sup>10–12</sup> are found in hormones, cytokines, transcription factors, RNA-binding proteins and enzymes. Higher-order antiparallel helical bundles are present in proteins such as TolC, whose structure includes an elongated 12-helical bundle.<sup>13,14</sup>

An important step in *de novo* protein design is the development of a mathematical model that defines the backbone conformation of the structure.<sup>6,15–17</sup> While the parameterization of coiled coils has been shown to be very useful for the design of parallel coiled coils,<sup>6,8,15,18</sup> less has been accomplished in the area of antiparallel helical bundles. We recently developed a parameterization of antiparallel four-helical bundles, which provided an excellent starting point for the design of diiron proteins.<sup>19,20</sup> Here, we extend this parameterization to include a variety of structures that conform to  $D_n$  symmetry.  $D_n$ -symmetrical bundles have an  $n$ -fold rotational symmetry axis directed through the center of the bundle, as well as  $n$  2-fold axes of rotational symmetry lying orthogonal to the bundle axis (Figure 1). Because of the orthogonal 2-fold axes, neighboring helices in  $D_n$ -symmetrical structures are oriented antiparallel to one another. Thus, for example, the application of a  $C_4$  symmetry operator to a single helix would result in a parallel four-helix bundle, while the application of a  $D_2$  symmetry operator results in an antiparallel four-helix bundle. Here, we consider three natural proteins whose structures show approximate  $D_2$ ,  $D_3$ , and  $D_6$  symmetry. In each case, the proteins have a cavity at the center of the structure that is capable of accommodating cofactors or substrates. Thus, these  $D_n$ -symmetrical bundles have the form of a tube.

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**Figure 1.** Side and top view of (a) four-helix bundle, (b) six-helix bundle, (c) 12-helix bundle with  $D_2$ ,  $D_3$  and  $D_6$  symmetry respectively, showing the  $n$ -fold rotational symmetry axis aligned with the bundle and 2-fold axes of rotation orthogonal to the bundle axis. The helices are represented as ribbons; the N termini and the C termini are in blue and red, respectively, showing the antiparallel arrangement of the helices due to the 2-fold rotational symmetry.

## Results

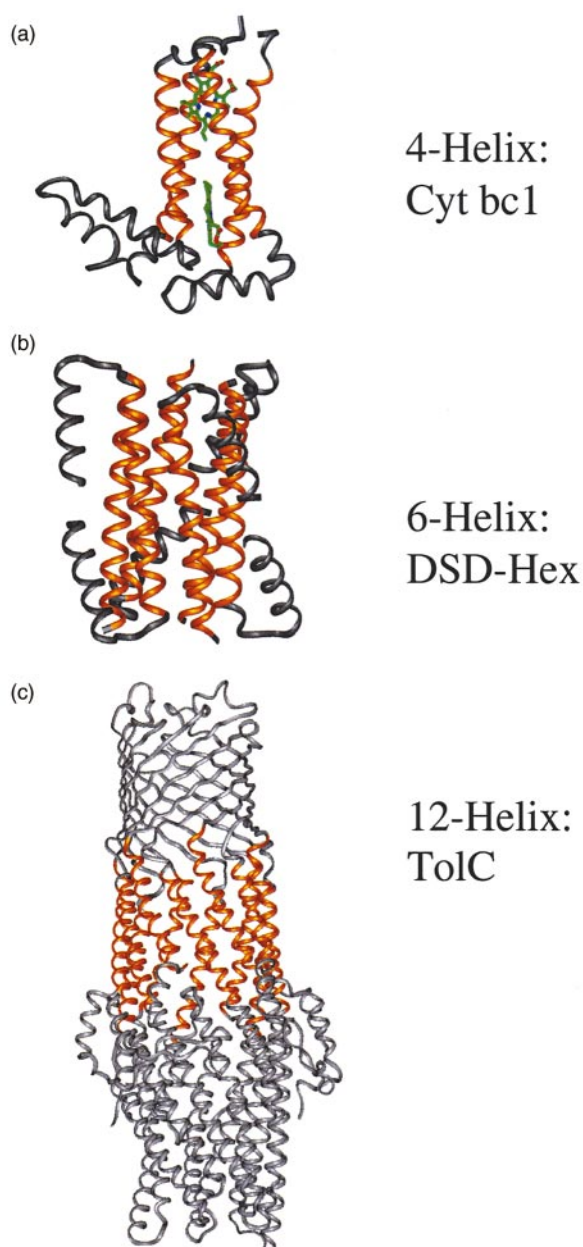
### $D_2$ symmetry

Four-helical bundles often show approximate  $D_2$  symmetry. Recently, we showed that models built with this symmetry reproduced the backbone geometries of six naturally occurring diiron proteins to a precision of approximately 1 Å r.m.s.d. Here, we extend this model to the central four-helical bundle in cytochrome  $bc_1$ <sup>21,22</sup> (Figure 2(a)), as an example of a heme-containing membrane protein. The core of  $bc_1$  contains two bis-His ligated hemes, which are important for shuttling electrons across phospholipid membranes. These hemes are sandwiched between two diagonally opposed helices, which each donate two His ligands to the heme-binding

sites. Previously, it was noted that this protein has quasi 2-fold symmetry with a helical pair as the asymmetric unit.<sup>21,22</sup> At the level of the backbone, the central four-helical bundle is well described by an even higher symmetry operator,  $D_2$ , with only a single helix as the asymmetric unit. Using a straight-helix model the r.m.s.d. is 1.16 Å with a total of 66 residues considered in the superposition. A coiled helix model results in an even better fit (Table 1) of 0.77 Å r.m.s.d with 85 residues included in the superposition (Figure 3(a)).

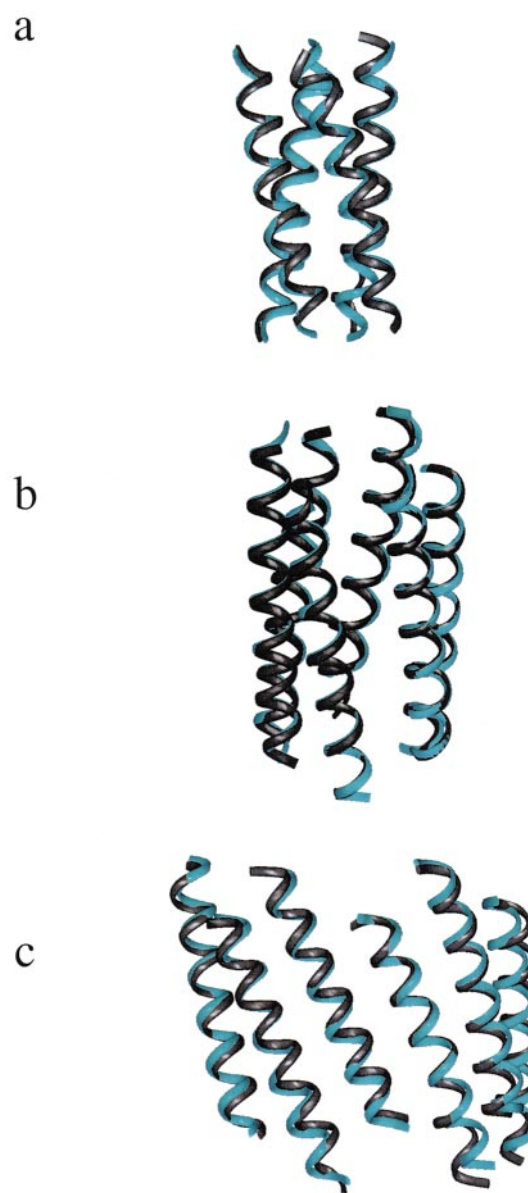
### $D_3$ symmetry

As an example of  $D_3$  symmetry, we considered the crystal structure of DSD,<sup>24</sup> a designed, hexame-



**Figure 2.** Experimental crystal structures of  $D_n$  helix bundles: the sections utilized for this work are highlighted in tan. (a)  $D_2$ , heme binding four-helix bundle in  $bc_1$ ; (b)  $D_3$ , six-helix bundle at the core of the hexameric assembly of DSD; (c)  $D_6$ , a 12-helix bundle forms the tubular structure of TolC.

ric protein (Figure 2(b)). This protein was designed to form a domain-swapped dimer consisting of two identical three-helix bundles. In dilute aqueous solution, the protein indeed forms the desired domain-swapped dimer. However, at protein concentrations above approximately 2 mM these dimers show weak higher-order association, and a hexamer was observed from the crystal structure of the protein.



**Figure 3.** Superposition of coiled helix backbone models (blue) generated as described in Methods to experimental crystal structures (gray); (a)  $D_2$ -symmetrical four-helix bundle and  $bc_1$ ; (b)  $D_3$ -symmetrical six-helix bundle and DSD; (c)  $D_6$ -symmetrical 12-helix bundle and TolC (for clarity, only half of the model is shown).

The unit cell of DSD contains three copies of a dimer, which forms the asymmetric unit in the crystal structure. The structure of the dimer is very close to that specified in its design. Each monomer contains a long helix, which packs against a second long helix from a neighboring subunit. N-terminal to the long helix is a second, shorter helix, which packs against the interface formed by the two long helices (Figure 2(b)). The two monomers in the dimer are related by approximate non-crystallographic symmetry (the r.m.s.d. of the backbone

**Table 1.** Parameters describing  $D_n$ -symmetrical coiled coils

Parameter	$bc_1$	DSD	TolC
$r_1$ (Å)	2.21	2.28	2.24
$\omega_1$ (deg./residue)	-102.32	-102.37	-103.03
$h$ , rise/residue (Å)	1.516	1.503	1.524
$\phi_1$ (deg.) <sup>a</sup>	8.5	3.6	5.7
$r_0$ (Å)	7.4	9.4	18.6
$P$ (Å)	171	288	308
$\phi_0$ (deg.) <sup>b</sup>	13	10	-0.2
$z_{trans}$ (Å)	0.47	1.81	3.65
Pitch angle $\alpha$ (deg.) <sup>c</sup>	15.3	11.6	20.7
Residues/turn (minor helix)	3.52	3.52	3.49
Residues/turn ( $\alpha$ -helix) <sup>d</sup>	3.63	3.58	3.55
Number of residues included in fit	85 <sup>e</sup>	156 <sup>f</sup>	300 <sup>g</sup>
r.m.s.d. (Å)	0.77	0.27	1.10

<sup>a</sup>  $\phi_1$  describes the rotation of the minor helix about its own axis. It is given relative to an "ideal" value in which a vector originating at the center of the helix and passing through the C $\alpha$  atom of the  $\alpha$  position is directed precisely towards the superhelical axis.

<sup>b</sup> The phase of the superhelix,  $\phi_0$ , is expressed relative to the value required to provide equal spacing between each neighboring helix.

<sup>c</sup> The pitch angle  $\alpha$  is calculated from the inverse sine of the circumference of the major cylinder *versus* its pitch.

<sup>d</sup> The number of residues per turn of the  $\alpha$ -helix is obtained by solving equation (1) for  $\omega_1$ .

<sup>e</sup> Residues C34-C52, C84-C105, C113-C134 and C183-C204 of the protein (pdb code, 1BCC) were included in the fit.

<sup>f</sup> Residues 21-46 and 71-96 from each of the subunits (as defined in pdb code, 1G6U) were included in the fit.

<sup>g</sup> Residues 15-39, 82-106, 221-245, and 300-324 of each of the subunits (pdb code, 1EK9) were included in the fit.

atoms is 0.16 Å). The long helices of the individual dimers further associate into a hexameric barrel-like structure, with the short helices packed against the outside of the barrel (Figure 2(b)). The central antiparallel six-helical bundle is very well described by  $D_3$  symmetry, with the coiled coil model (Figure 3(b)) again providing a better fit (0.27 Å, Table 1) than the straight helix model (0.79 Å).

### $D_6$ symmetry

TolC is one component of a bacterial transport system that is responsible for efflux of selected molecules from *Escherichia coli*.<sup>14</sup> TolC forms a long 12-stranded coiled coil, which forms a water-soluble extension of a porin-like transmembrane domain. The membrane-proximal half of this bundle forms a very regular barrel-like structure, while the packing deviates from this idealized geometry near the bottom of the protein as viewed in Figure 2(c). The TolC 12-helical bundle consists of a crystallographic trimer of four non-identical helices. It has been noticed that the structure has approximate  $C_6$  symmetry with a two-helix structure in the asymmetric unit.<sup>13</sup> The N-terminal portion of the bundle (300 residues) can also be described by a  $D_6$  symmetrical model (Figure 3(c)) with a single helix in the asymmetric unit. Again, a bundle with coiled helices fits better (1.10 Å r.m.s.d.) than a model with straight helices (1.24 Å r.m.s.d.).

### Location of cofactors and bound ions

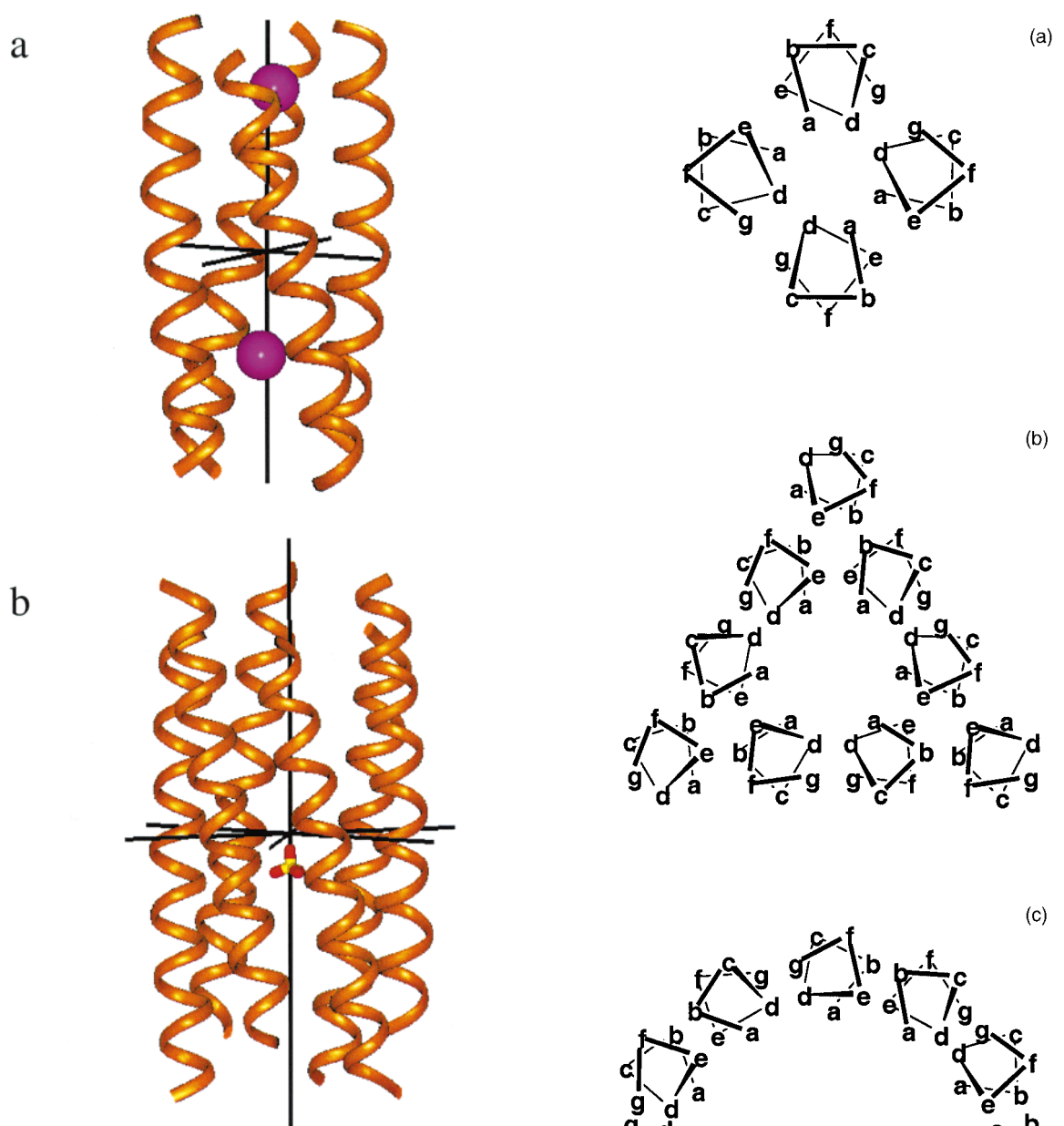
The approximate  $D_n$  symmetry of these proteins extends to the positions of bound cofactors and ions. In DSD, a sulfate ion is located along the crystallographic 3-fold axis 2.8 Å from the origin of

the structure (Figure 4(b)). In  $bc_1$  the heme iron atoms lie 0.5 Å and 2.8 Å from the central superhelical axis, respectively. However, the placement of the iron atoms relative to the origin of the structure is more consistent with  $C_n$  than  $D_n$  symmetry; one iron atom is located 8.1 Å above and one is located 12.2 Å below the origin (Figure 4(a)). The relatively large deviation from  $D_n$  symmetry arises from the fact that the His side-chains are located on only two, rather than all four of the helices.

### Organization and packing in $D_n$ -symmetrical bundles

Figure 5 illustrates idealized geometries for  $D_2$ ,  $D_3$  and  $D_6$ -symmetrical bundles. Each position in the heptad repeat is labeled in the usual nomenclature for coiled coils,<sup>1</sup> with the residue that projects most directly towards the central axis of the bundle being defined as a. In each case, the proteins show two geometrically distinct helix-helix pairings. In the  $D_2$ -symmetrical bundle (Figure 5(a)), the packing of residues at positions a and e from neighboring helices define one such interface (defined here as the a/e interface); residues at d and g pack at the other interface, denoted the d/g interface.

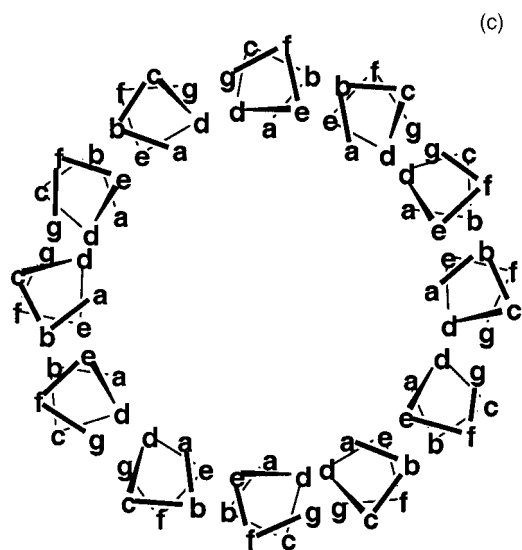
In the  $D_6$ -symmetrical structure (Figure 5(c)), one helix-helix interface is primarily defined by the packing of residues at b and e, while residues at d and g define the second interface. The structure of the  $\alpha$ -helical coiled coil appears to be well suited to the formation of 12-sided structures; consider two lines running through the center of a helix and bisecting either b and e or d and g. These two lines will cross at an angle of 154° ( $3/7 \times 360^\circ$ ), which is very close to the angle of 150°, required for the generation of an equilateral dodecagon. This arrangement has also been noted by Walshaw &



**Figure 4.** Position of bound heme iron ions in  $bc_1$  (a) and sulfate ion in DSD (b) with respect to the symmetry axes.

Woolfson<sup>24</sup> as well as by Calladine *et al.*<sup>25</sup> How then, is the six-sided  $D_3$  bundle formed?

The  $D_3$  bundle (Figure 5(b)) in DSD is a trimer of domain-swapped, three-helical bundles. The three-helix bundles are stabilized by a packing interaction between three helices, whereas the packing between different three-helix bundles involves a two-helix interface. Thus, as one progresses around the  $D_3$ -symmetrical inner ring of helices, each helix alternately forms a two-helix interaction along its d-g surface and a three-helix interaction along its b-e surface. As discussed,<sup>5</sup> different geometries are



**Figure 5.** Helical wheel diagrams of antiparallel helix bundles, describing side-chain positions in the heptad repeat for (a)  $D_2$ , (b)  $D_3$  and (c)  $D_6$ .

observed for two *versus* three-helix bundles, with “acute” packing being observed in the three-helix bundle. This difference in helix packing angles, together with small differences in helix-packing

radii, provide a hexameric rather than a 12-sided structure.

### Comparison of the structural parameters describing $D_n$ -symmetrical coiled coils

Table 1 summarizes the relevant parameters defining the three types of coiled coils investigated in this work. In each structure, the minor helix has a nearly perfect 3.5-residue repeat, as expected for coiled coils. This observed 3.5-residue repeat is a result of applying a left-handed superhelical twist to an  $\alpha$ -helix. Dunker & Zaleske<sup>26</sup> discussed the relationships between the pitch ( $P$ ), superhelical radius ( $r_0$ ), and the pitch angle, ( $\alpha$ , the dihedral angle between the minor and major helical axes) of a coiled coil. The pitch angle is defined by equation (1):

$$\alpha = \tan^{-1}[(\omega_1 - \omega_\alpha)(r_0)/(h)] \quad (1)$$

in which  $\omega_1$  is the minor helical angular frequency in its own reference frame (which rotates about the superhelical axis) and  $\omega_\alpha$  is the  $\alpha$ -helical angular frequency in the global (fixed) reference frame. Both are expressed in radians per residue ( $2\pi/3.5$  for an ideal coiled coil, and  $2\pi/3.62$  for an idealized  $\alpha$ -helix). The parameter  $r_0$  is the radius of the superhelix, and  $h$  is the rise per residue of the alpha-helix. For coiled coils with relatively small radii, idealized values for the geometric parameters of the  $\alpha$ -helix can be used to predict the value of  $\alpha$ . For example, the value predicted for  $bc_1$  ( $r_0 = 7.4 \text{ \AA}$ ) is  $16.3^\circ$  using 3.5 residues/turn for the coiled coil minor helix, 3.62 residues/turn for the  $\alpha$ -helix, and  $h = 1.51 \text{ \AA/residue}$ . This value is in very good agreement with the observed value of  $15.3^\circ$ , and this pitch angle allows highly favorable interactions between the side-chains of neighboring helices. However, as the radius of the helix is increased, the predicted pitch angle increases beyond the range of values that are consistent with reasonable helical geometries and inter-helical packing interactions. For example, the value predicted for the TolC bundle ( $r_0 = 18.6 \text{ \AA}$ ) is  $36.2^\circ$ , considerably greater than the observed value of  $20.7^\circ$ . Instead, it has been predicted<sup>26</sup> that as the radius of a coiled coil is increased, the  $\alpha$ -helices will slightly unwind, thereby providing a more reasonable value of  $\alpha$ . Indeed, as the radii of the bundles in Table 1 increase from  $7.4 \text{ \AA}$  to  $18.6 \text{ \AA}$ , the repeat of the  $\alpha$ -helix decreases from 3.63 residues/turn to 3.55 residues/turn. A similar value of 3.54 was calculated for the TolC bundle by Calladine *et al.*<sup>25</sup> using a different method. This deviation from an ideal  $\alpha$ -helical geometry appears to represent a relatively low-energy deformation, based on the fact that helices with 3.5 to 3.6 residues/turn occur quite frequently in globular proteins.<sup>27</sup>

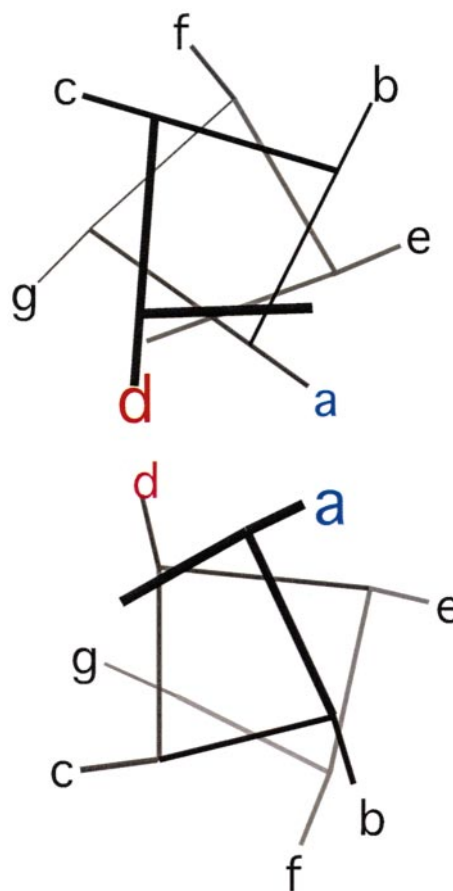
A second consequence of the unwinding of the  $\alpha$ -helix is that the pitch of the superhelix changes according to equation (2):

$$P = [(2\pi h/(\omega_1 - \omega_\alpha))^2 - (2\pi r_0)^2]^{1/2} \quad (2)$$

The first term in equation (2) increases with increasing radius (because of the concomitant decrease in the difference between  $\omega_1$  and  $\omega_\alpha$ ), while the second term becomes more negative with increasing  $r_0$ . In this series of bundles, the first term dominates; as  $r_0$  changes from  $7.4 \text{ \AA}$  in  $bc_1$  to  $18.6 \text{ \AA}$  in TolC,  $P$  increases from  $171 \text{ \AA}$  to  $308 \text{ \AA}$ .

### Helix pairwise packing interactions

It is instructive to consider the similarities and differences in the geometries of the helix-helix packing interactions in  $D_n$ -symmetrical bundles. To allow a comparison of the interhelical packing arrangements in proteins with different symmetry, it is useful to introduce a heptad nomenclature that depends only on the relationship of the two interacting helices, and is independent of the aggregation number of the bundle. In this convention, the residues projecting towards the neighboring helices are designated  $a'$  and  $d'$ , respectively (Figure 6). Thus, if the b-e interface of the  $D_6$  bundle



**Figure 6.** Helical wheel diagram illustrating the side-chain interactions between a pair of neighboring helices in antiparallel helix bundles. The residues projecting towards the helix-helix interface are designated  $a'$  and  $d'$ , respectively, independent of their position in the parent bundle.

(Figure 5(c)) is considered as a pairwise interaction, the b and e positions become a' positions and d' positions, respectively. Similarly, the d and g positions of a d-g interface become a' and d', respectively, in the pairwise nomenclature.

Figure 6 displays a typical antiparallel helical pair with the positions labeled a' through g'. The helical pair can be divided into two halves, with positions a', b', and e' on one side of the structure, and positions d', g' and c' on the opposite side. Following the convention of Efimov,<sup>28</sup> if residues a', b' and e' face the interior of the protein, the helix pair is defined as left-handed; the pair is considered right-handed if d', g' and c' face the interior of the bundle.  $D_n$ -symmetrical bundles have an equal number of left-handed and right-handed helical pairs, forming a closed, circular structure when viewed from the top of the bundle (Figure 5).

Table 2 provides the interhelical distances and packing angles, associated with the helical pairs in the  $D_n$ -symmetrical bundles. Because a rotational axis relates the two individual helices in each pair, only two angles and two translations are required to approximately characterize their geometries: the interhelical distance  $d$ , the interhelical packing angle  $\delta$ , the rotation of the helix about its own axis,  $\epsilon$ , and the vertical displacement of the helices,  $d_z$ . In Table 2, the value of  $d_z$  is also expressed as the distance between the a residues expressed in fractions of a heptad, as described.<sup>29</sup> The angle  $\epsilon$  is defined by measuring the angle between a line connecting the center of the two helices and a vector from the center of an individual helix that bisects its a' and d' C $\alpha$  atoms.

The values of the interhelical distance and the packing angles are reasonably well clustered for four of the six interfaces described in Table 2, including both interfaces of TolC, the a-e interface of  $bc_1$ , and the b-e interface of DSD. These helical pairs each represent variants of an Alacoil  $\alpha$ -packing motif, in which Ala residues (or other small side-chains) occupy either the a' or d' position of

the helix.<sup>29</sup> A particularly striking example of a "ferritin-like" Alacoil is encountered in the d-g interface of DSD (Figure 7(a)). In this Alacoil, the Ala residues occur in the a' positions on the inner side of the barrel. Their small size allows efficient packing of the helices, resulting in a separation of 8.0 Å. A second ROP-like Alacoil, with the Ala side-chains occurring at the d' positions, is found in a d-g interface of TolC (Figure 7(b)). As described by Gernert and co-workers,<sup>29</sup> the offset of the helices is different for the ferritin-type *versus* the ROP-type packing (Figure 7(b)). This difference in  $z_{trans}$  is a result of the fact that the C $\alpha$ -C $\beta$  bonds do not radiate directly outward from the helix, when viewed axially (see Figure 6). Also, the C $\alpha$ -C $\beta$  bonds are angled towards the N terminus of the helix when viewed from the side of the helix (not shown). Calladine *et al.*<sup>25</sup> and Walshaw & Woolfson<sup>24</sup> have conducted a very extensive analysis of side-chain packing in TolC, and the role that sterics play in fine-tuning the geometry of the helix-helix interface.

Two helical interfaces deviate significantly from the others in terms of their packing parameters (Table 2). The  $bc_1$  protein has one helix-helix interface with a relatively long (11.6 Å) interhelical distance and large values of  $\delta$  and  $\epsilon$ , which result from the insertion of the heme between the two helices. There is relatively little interaction between the helices along this interface, which is dominated by the interactions of the helices with the porphyrin ring. In contrast, the helices interact extensively along the d-g interface of  $bc_1$ , in a variant of the Alacoil motif in which the helices are slightly further apart than in a canonical structure.

The b-e interface of DSD is involved in a three-helix bundle interaction that gives rise to differences in its inter-helical packing parameters. The core of the three-helix bundle is packed with Leu side-chains, which gives rise to a relatively large inter-helical separation (10.8 Å). The acute inter-helical packing angle found in three-helix bundles<sup>5</sup>

**Table 2.** Interhelical packing distances and angles

Interface	a' residue <sup>a</sup>	$d$ (Å) <sup>b</sup>	$d_z$ (Å) <sup>c</sup>	$\delta$ (deg.) <sup>d</sup>	$\epsilon$ (deg.) <sup>e</sup>
$bc_1$ (a-e interface)	e	9.2	2.0 (0.19)	18.9	3.5
$bc_1$ (d-g interface)	d	11.6	4.9 (0.48)	23.9	15.8
DSD (b-e interface)	b	10.8	0.69 (0.067)	13.4	17.6
DSD (d-g interface)	d	8.0	3.7 (0.36)	9.7	-14.3
TolC (b-e interface)	b	9.6	1.3 (0.13)	10.5	-4.4
TolC (d-g interface)	d	9.8	4.4 (0.45)	10.6	-8.4

The distances and angles are defined by first orienting the bundles in a Cartesian coordinate system with the superhelical axis along  $z$ , and the orthogonal 2-fold axes in the  $x$ - $y$  plane.

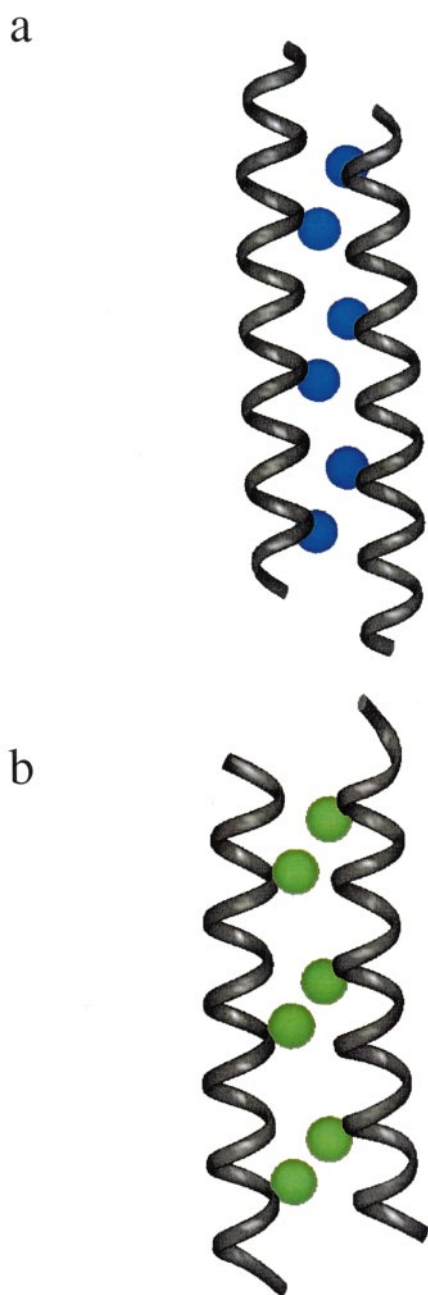
<sup>a</sup> The residue in the heptad nomenclature used in Figure 6, corresponding to the a' position in the pairwise helical nomenclature.

<sup>b</sup>  $d$  is the distance of closest approach between the axes of the individual helices.

<sup>c</sup>  $d_z$  is the distance along the  $\alpha$ -helical axis from the  $x$ - $y$  plane to the closest a' residue. The values in parentheses are expressed in fractional distance along a heptad unit, following the convention of Gernert *et al.*<sup>29</sup>

<sup>d</sup>  $\delta$  is the interhelical crossing angle defined by the dot product between tangents to the helical axes of neighboring helices. It is defined to be zero when the helices are antiparallel.

<sup>e</sup>  $\epsilon$  describes the rotation of the helix about its own axis. It is given relative to an "ideal" value expected if a vector originating at the center of the helix and bisecting the C $\alpha$  atoms of the central a' and d' positions (in a projection onto the  $x$ - $y$  plane) were directed precisely towards the axis of the neighboring helix. The angle is measured at the heptad position closest to the  $x$ - $y$  plane.



**Figure 7.** Side view of Alacoil motifs in DSD and TolC. (a) In DSD the Ala side-chains lie at the a' positions, forming a ferritin-type Alacoil. (b) A ROP-type Alacoil motif with Ala side-chains at d' positions is found in one of the two interfaces of TolC.

is reflected in a value of  $\epsilon$  angle that is larger from the values observed for the other helix-helix packings (Table 2).

## Conclusions

The parameterization of protein three-dimensional structures is an important tool for *de novo*

protein design. A very early attempt at protein design used a  $D_2$  symmetry operator to define the backbone structure of a four-helical bundle.<sup>30–32</sup> Although this protein was subsequently found to adopt a molten globule conformation,<sup>17</sup> refinements in side-chain packing<sup>33–35</sup> have made this general approach more suitable for *de novo* protein design of complex, functional proteins.<sup>20,36</sup> Here, we demonstrate that a very simple mathematical formalism is able to capture the essential features of the structures of several complex proteins. The  $D_n$ -symmetrical model used in this work is particularly well suited to the analysis and design of tubular proteins that contain binding sites within their central cores. Depending on the aggregation number, the tubes may contain tightly packed cofactors, as in  $bc_1$  and diiron proteins,<sup>19,20</sup> or large cavities capable of accommodating a variety of substrates as in TolC. The parameters that have been obtained from these models can be used for the design of idealized versions of these folds, or they can be varied to allow the design of entirely novel structures.

In automated approaches to protein design, the parameters defining the backbone structure are allowed to vary, providing a solution to the flexible docking problem.<sup>6,15,16,33–35,37</sup> In this manner, it is possible to search both conformational space as well as sequence space to find a sequence that can stabilize a given fold. The use of  $D_n$ -symmetry operators should be particularly valuable in the initial phases of computational design. Because a specific function often requires a loss of symmetry, the  $D_n$  symmetry of the original bundle can be lifted as the design progresses, as in our previous work on diiron proteins.<sup>20</sup> The design of a variety of  $D_2$ -symmetrical heme-binding proteins,  $D_3$ -symmetrical hexamers, and  $D_6$ -symmetrical tubes is in progress, and will be reported elsewhere.

An interesting finding of this and other papers<sup>24,25</sup> is the observation that the helical faces used in the helix-helix packings help to define the aggregation number of the bundle. The a-e and d-g interfaces are oriented at nearly  $90^\circ$  angles, fitting nicely with the rectangular cross-section of an antiparallel four-helix bundle. Similarly, the b-e and d-g interfaces subtend a wider angle, nearly ideal for a 12-helix bundle. Finally, by alternating two-helix and three-helix interfaces, it is possible to engineer a hexameric bundle. The actual angles within the bundle are further fine-tuned by the nature of the steric interactions between the side-chains.<sup>26</sup> It would be interesting to determine whether further modifications in the bulk of the interacting side-chains could result in a change in the aggregation number, similar to the changes observed in variants of GCN4 P1<sup>4</sup>.



## Methods

### Fitting idealized backbones to experimental structures

We examined two mathematical models to describe the backbone conformations of  $bc_1$ , DSD, and TolC. The first is a  $D_n$ -symmetrical bundle of straight  $\alpha$ -helices,<sup>19</sup> while the second uses a coiled coil model.<sup>38</sup> The fitting parameters for the first method were the three Eulerian rotations and the three translations required to define the location of a monomeric helix within the coordinate system. A modification of the Crick parameterization was used for the coiled-coil model, which employs six parameters to define the path of an individual chain: the radius ( $r_1$ ), angular frequency ( $\omega_1$ ), rise per residue ( $h$ ) and phase ( $\phi_1$ ) of the minor helix, as well as the radius ( $r_0$ ), and pitch ( $P$ ) of the superhelix. While these parameters suffice to describe a  $C_n$ -symmetrical coiled coil, two additional parameters are necessary to describe the interhelical orientations of antiparallel  $D_n$ -symmetrical coiled coils. These parameters include the phase of the superhelix ( $\phi_0$ ) and the translation of the supercoiled helix along the superhelical axis ( $z_{trans}$ ).

The parameters,  $r_0$ ,  $r_1$ ,  $P$ ,  $\omega_1$ ,  $\phi_1$ ,  $\phi_0$ ,  $h$ , and  $z_{trans}$  were fit to the  $C^\alpha$  coordinates of the proteins using the equations:

$$x = r_0 \cos \omega_0 t + r_1 \cos \omega_0 t \times \cos(\omega_1 t + \phi_1) - r_1(P/(2\pi r_0^2 + P^2)^{1/2}) \times \sin \omega_0 t \times \sin(\omega_1 t + \phi_1) \quad (3a)$$

$$y = r_0 \cos \omega_0 t + r_1 \sin \omega_0 t \times \cos(\omega_1 t + \phi_1) - r_1(P/(2\pi r_0^2 + P^2)^{1/2}) \times \sin \omega_0 t \times \sin(\omega_1 t + \phi_1) \quad (3b)$$

$$z = -Pt + r_1(2\pi r_0/(2\pi r_0^2 + P^2)^{1/2}) \times \sin(\omega_1 t + \phi_1) \quad (3c)$$

in which  $t$  is the residue number (for the examples in Table 1, the center residue was defined to be the zeroth position). The parameter  $\omega_0$  is the angular frequency of the major helix (in radians per residue). It is related to the fitting parameters according to equation (4):

$$\omega_0 = 2\pi h/(r_0^2 + P^2) \quad (4)$$

These equations generate a superhelix, which rotates around the  $z$ -axis. To generate the remaining helices, the superhelix is translated in the  $z$  direction by the value  $z_{trans}$ , rotated by  $\phi_0$ , and the appropriate symmetry operations are performed to generate the bundle.

For each system, the helical regions of the peptides were fit to equations (3) (Table 1). Eight parameters are fit using a genetic algorithm (the remaining parameters are derived from these variables). The algorithm encodes the eight parameters as a contiguous binary number, which is then modified by either swapping with a portion of another string or by randomly changing a single digit in the sequence, which is referred to as "mutation". One "immortal" chromosome, corresponding to the best fit in that generation, is passed unmodified to the next generation. The algorithm has a mutation rate of 0.08, a single crossover rate of 0.65, and a double crossover rate of 0 with the  $C^\alpha$  r.m.s.d. fit used as a scoring function. The algorithm is stopped once the fitting parameters and the r.m.s.d. are constant for at least 20 generations. Tests on fitting the  $bc_1$  peptide indicate that after 20 gener-

ations of being constant, the fit does not change after 150 additional generations. Once the alpha carbon atoms are in place, the rest of the backbone is generated by assuming that the peptide is approximately helical. This preliminary backbone is then refined (with fixed alpha carbon positions) through energy minimization using the cvff force-field in the program Discover.

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