Structural basis for antibiotic recognition by the TipA class of multidrug-resistance transcriptional regulators

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The TipAL protein, a bacterial transcriptional regulator of the MerR family, is activated by numerous cyclic thiopeptide antibiotics. Its C-terminal drug-binding domain, TipAS, defines a subfamily of broadly distributed bacterial proteins including Mta, a central regulator of multidrug resistance in *Bacillus subtilis*. The structure of apo TipAS, solved by solution NMR [Brookhaven Protein Data Bank entry 1NY9], is composed of a globin-like α -helical fold with a deep surface cleft and an unfolded N-terminal region. Antibiotics bind within the cleft at a position that is close to the corresponding heme pocket in myo- and hemoglobin, and induce folding of the N-terminus. Thus the classical globin fold is well adapted not only for accommodating its canonical cofactors, heme and other tetrapyrroles, but also for the recognition of a variety of antibiotics where ligand binding leads to transcriptional activation and drug resistance.

Keywords: antibiotic recognition/globin fold/heteronuclear NMR/protein dynamics/transcriptional regulation

Introduction

Multidrug resistance (MDR) systems in bacteria often rely on transport proteins or modifying enzymes to exclude or inactivate a large variety of different antimicrobial xenobiotics and transcriptional regulatory proteins having a comparable recognition spectrum (Grkovic *et al.*, 2001). The recent crystal structures of the *Bacillus subtilis* MDR-regulator BmrR (Zheleznova *et al.*, 1999; Heldwein and Brennan, 2001) and of other regulators (Aleksun *et al.*, 2001; Schumacher *et al.*, 2001, 2002) have yielded important insights into the structural basis of this multidrug recognition. BmrR belongs to the family of MerR-like proteins, characterized by their homologous N-terminal DNA-binding domains and related mechanisms of transcriptional activation. Various members of this family respond to general stress-response signals, specific

molecules or multiple drugs. In addition to MerR, the mercury resistance regulator protein (Miller, 1999), well-characterized representatives include the superoxide resistance regulator SoxR (Pomposiello and Demple, 2001), and the MDR transcriptional regulators BmrR (Ahmed *et al.*, 1994), BltR (Ahmed *et al.*, 1995) and Mta (Baranova *et al.*, 1999).

The TipAL protein (Murakami *et al.*, 1989) represents another distinct member of the MerR family first identified in *Streptomyces*, organisms recognized as the producers of most natural antibiotics. The *tipA* gene can confer resistance to the antibiotic thiostrepton in *Streptomyces lividans*. Its transcription is induced by a large family of antibiotics produced by *Streptomyces*, *Bacillus* and *Micrococcus* including thiostrepton, nosiheptide and promethiocin (Figure 1). These compounds are highly modified cyclic thiopeptides with an attached linear peptide 'tail' containing at least one dehydroalanine. Thiostrepton inhibits protein synthesis by forming stable ternary complexes with the 23S ribosomal RNA and the ribosomal protein L11 (Hill *et al.*, 1990; Porse *et al.*, 1998). Thus thiostrepton has been used as a tool to study ribosomal function (Hill *et al.*, 1990).

In response to thiostrepton and other antibiotics of its class, the *tipA* gene is expressed in *S.lividans* as two alternate in-frame translation products: a long form, TipAL (253 amino acids), and a short form, TipAS (144 amino acids), which constitutes the C-terminal part of TipAL (Holmes *et al.*, 1993). Owing to its high sequence homology with other MerR-like proteins, the N-terminal domain of TipAL (residues M1–S109; Figure 2) is predicted to include a helix–turn–helix motif (V5–I25), responsible for DNA binding, and a long coiled-coil dimerization motif (A74–S109). TipAS (residues M110–P253) forms the antibiotic-recognition domain. The variable C-terminal sequences of the MerR protein family correspond to different ligand-binding modules tailored to specific targets. In fact, ligands recognized by MerR proteins range from divalent metal ions to large antibiotics such as thiostrepton.

Antibiotic binding to TipAS or TipAL is irreversible due to the formation of a covalent bond between cysteine 214 and a dehydroalanine residue of the antibiotic (Chiu *et al.*, 1996). Binding of thiostrepton increases the affinity of TipAL for its operator site and induces recruitment of RNA polymerase (RNAP) to the promoter *ptipA* (Chiu *et al.*, 2001). TipAL and other MerR-type regulators bind as dimers to an inverted repeat sequence located within the spacer region of their promoters. By analogy with other MerR proteins, the ligand-bound TipAL dimer may activate transcription by twisting the DNA helix. This twisting of the promoter brings the two consensus recognition sequences into linear alignment, thus allowing RNAP to initiate transcription (reviewed in

Fig. 1. Chemical structures of the *tipA*-inducing thiopeptide antibiotics thiostrepton, nosiheptide and promothiocin A. All *tipA* inducers are bacterial secondary metabolites with cyclic structures built from highly modified amino acids. The shaded area indicates a part of the first macrocycle which is conserved among the three antibiotics (see text).

Summers, 1992). Recently, the crystal structure of a BmrR–ligand–*bmr* promoter complex (Heldwein and Brennan, 2001) has provided an elegant visualization of this DNA distortion and has suggested that drug binding causes the repacking of a helix within the mixed α – β structure of the BmrR drug-recognition domain.

In *S.lividans* cultures induced with thiostrepton, TipAS is expressed in large molar excess over TipAL (>20:1) (Holmes *et al.*, 1993). Sequestering of the drug by TipAS not only confers resistance by neutralizing the bulk of antibiotic in the cytosol but also limits a drug-dependent positive feedback loop that provides for autogenously regulated expression. However, besides controlling its own expression, TipA may be involved in the regulation of other MDR genes in *S.lividans*. Although the respective genes are not yet defined, the inducer of TipA, thiostrepton, also induces resistance to several unrelated antibiotics in *S.lividans* (Guilfoile and Hutchinson, 1991; Plater and Robinson, 1992). Thiostrepton enhances the synthesis of several polypeptides (Murakami *et al.*, 1989; Holmes *et al.*, 1993) with at least one of them dependent on the presence of an intact *tipA* gene.

The entire TipAL sequence, including its TipAS domain, has significant similarities not only to the known homologs Mta in *B.subtilis* (Baranova *et al.*, 1999), the regulator protein for PmrA in *Streptococcus pneumoniae* (Tettelin *et al.*, 2001) and the SkgA protein of *Caulobacter crescentus* (Rava *et al.*, 1999), but also to many other proteins in pathogenic and common environmental bacteria. Mta stimulates expression of the multi-drug-efflux transporter genes *bmr* and *blt* (Baranova *et al.*, 1999) and thereby activates a regulon of drug transporter genes. The Mta-activating ligands are unknown. However, they may be related to cyclic peptides recognized by TipAS. TipAS-like proteins do not share significant sequence homologies with any currently known protein structure.

Here we report the three-dimensional NMR structure of apo TipAS and the changes induced by complex formation with the antibiotic. The binding event induces folding of the unstructured N-terminal part of apo TipAS, which forms the linker to the DNA-binding domain in TipAL.

This observation suggests that the folding of the linker and its mechanical interactions with the DNA-binding domain may be responsible for the increased affinity of ligand-bound TipAL to the *tipA* promoter, the DNA twisting and the subsequent recruitment of RNAP. Similar modes of ligand recognition and possibly function are expected for a large class of homologous bacterial MDR regulator proteins. Unexpectedly, it was found that the all-helical fold of TipAS and its ligand recognition site are similar to hemoglobin and other members of the globin family. This finding raises interesting questions about the evolution of the globin fold and its ligand-binding properties.

Results and discussion

The drug-binding domain of TipAL is present in a wide variety of environmental and pathogenic bacteria

While the N-terminal DNA recognition motif of TipAL was known to have sequence homologies to the very large group of MerR-like proteins, a survey of recently described sequences revealed that the entire TipAL protein, including its C-terminal drug-binding domain, defines a new subfamily (Figure 2). MerR-like proteins with a C-terminal drug-binding domain homologous to TipAS are present in a wide variety of bacteria. Such proteins have been studied in some detail in *B.subtilis* (Mta, 40% total sequence identity; Baranova *et al.*, 1999; Takami *et al.*, 2000), *S.pneumoniae* (the regulator protein for PmrA, 33% identity; Tettelin *et al.*, 2001) and *C.crescentus* (SkgA, 32% identity; Rava *et al.*, 1999). In addition to these published sequences, a BLAST search (Altschul *et al.*, 1990) of the database of unfinished genomic sequences at The Institute for Genomic Research (TIGR) (<http://www.tigr.org>) yielded further homologs of the TipAS sequences in the genomes of many pathogenic bacteria, including *Bacillus anthracis* (39% total sequence identity with TipAL), *Streptococcus mitis* (32% identity), *Enterobacter faecalis* (32% identity) and *Listeria monocytogenes 4b* (36% identity), as well as common environmental species such as *Mycobacterium smegmatis* (44% identity) and *Bacillus halodurans* (36% identity).

Fig. 2. Sequence alignment of the TipAL sequence with homologs from the family of MerR-like proteins. Alignment is shown for the C-terminal TipAS-like part of the proteins. Positions with high amino acid similarity are indicated by red letters and strictly conserved residues by red rectangles. Yellow indicates the initial methionine of TipAS (residue M110 of TipAL) and nearby methionines in the homologs, which potentially serve as sites for translational initiation. A red line emphasizes their respective positions. Positions of TipAS α -helices determined in this study are indicated above the alignment as magenta cylinders. Open cylinders denote helices that are only stable in the presence of ligand. The N-terminal, DNA-binding part of TipAL is highly conserved among all MerR-like proteins. This part of the sequence is only shown for TipAL. Arrows mark the position of the expected helix-turn-helix (H-T-H) motif and the putative coiled-coil dimerization region based on the structure of the MerR protein BmrR (Heldwein and Brennan, 2001). The alignment was generated by the program MultAlin (Corpet, 1988).

Most TipA homologs in the TIGR data base have not been studied in more detail. However, the characterized homologs in *B. subtilis* (Baranova et al., 1999; Takami et al., 2000) and *S. pneumoniae* (Tettelin et al., 2001) constitute transcriptional regulators of MDR transporters, and the SkgA protein of *C. crescentus* is a regulator of starvation-induced resistance to H_2O_2 (Rava et al., 1999). Thus, for all cases described, the TipAL homologs control resistance to drugs or toxins. The TipAL subfamily seems to share one additional feature: all the homologs mentioned above contain one methionine residue close to the position of M110 in TipAL, i.e. the N-terminus of TipAS (Figure 2). Therefore, it is possible that also in the other proteins of the TipAL subfamily, the C-terminal parts are translated as separate short forms.

NMR spectroscopy and determination of secondary structure elements

TipAS backbone and side-chain resonance assignments were carried out using standard triple-resonance NMR techniques as described in Materials and methods. The ^1H - ^{15}N -HSQC spectrum of TipAS (partially shown in Figure 3A) exhibits strong heterogeneity in the intensities

of the amide resonances. Although a fraction of the resonances are weak and partially broadened, the majority are strong and narrow, albeit not very well dispersed. During the sequential assignment process, it became clear that the weak resonances belong to the N-terminal 54 amino acids of TipAS, whereas the strong resonances correspond to C-terminal residues D165–T252. Assignment for the latter region is complete, with the exception of the partially assigned residues H204–C207, which also showed very weak resonances. The C-terminal region of TipAS forms an all-helical fold. Strong positive $^{13}C\alpha$ and small $^{13}C\beta$ secondary shifts (Spera and Bax, 1991), as well as a large number of sequential $d\alpha_N(i, i+3)$ and $d\alpha_N(i, i+4)$ nuclear Overhauser effect (NOE) connectivities (Wüthrich, 1986), define five α -helices within this part of apo TipAS (Figures 2 and 3C). The helices and respective residues are $\alpha 1$ (K157–D179), $\alpha 2$ (E187–N203), $\alpha 3$ (Y209–M218), $\alpha 4$ (E223–A232) and $\alpha 5$ (L236–H251).

In contrast to the C-terminal part, amide resonances in the N-terminal region that precedes residue D165 are mostly weak or even absent (Figure 3A). This situation could not be improved by a variation of the solvent

Fig. 3. NMR evidence for ligand-induced folding of TipAS. Comparison of the central region of ^1H - ^{15}N -HSQC spectra of apo TipAS (A) and TipAS bound covalently to the antibiotic promethicin A (B). Owing to intermediate exchange, a number of ^1H - ^{15}N correlations (dotted circles) are not visible in the apo TipAS spectrum. These resonances were only detected after considerably increased data acquisition times such as in three-dimensional HNCOC experiments. (C) Heteronuclear $\{^1\text{H}\}$ - ^{15}N NOE indicative of the variations in the pico- to nanosecond mobility of amide groups for apo TipAS (open circles) and the TipAS/promethicin A complex (filled diamonds). The position of the TipAS α -helices is indicated at the top. Filled helices are present in both the apo and the complexed form. Open helices are only formed in the complex.

conditions, i.e. pH, ionic strength or temperature. Within this N-terminal part of TipAS, 28 amino acids were assigned to stretches G111–T115, V122–F126, G140–Y145 and A153–Q164. For all the observed residues in the range of G111–Y145, the absence of NOE cross-peaks and small $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ secondary shifts indicate a random coil conformation. The weakness of the resonances in this region cannot be attributed to amide proton exchange with solvent because the pH of the experiments was comparatively low (5.9) and water flip-back techniques were used that greatly reduce such signal losses (Grzesiek and Bax, 1993). Therefore, we attribute the weak signal intensities to conformational exchange on the intermediate chemical shift timescale. Apparently, the backbone within this

region is not stable, and changes between different conformations occur within micro- to milliseconds.

The region of weak resonances extends to residue Q164 (Figure 3A), which is already part of helix $\alpha 1$ as defined by $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ secondary shifts (data not shown). This indicates that the N-terminus of helix $\alpha 1$ up to residue Q164 is also not completely stable. Only weak NOEs were detected within this part of helix $\alpha 1$. For this reason, the structure calculations were restricted to residues D165–P253, which form the well-folded part of apo TipAS.

The solution structure of TipAS

The solution structure of the folded part of apo TipAS shows a pairwise antiparallel arrangement of helices $\alpha 1$ and $\alpha 2$ versus helices $\alpha 3$ and $\alpha 5$ (Figure 4) that forms a large, cleft-like opening. A type I β -turn forms the connection between $\alpha 1$ and $\alpha 2$. Within this turn, residue A184 is completely buried in the hydrophobic core of the protein fold, thus fixing the entire turn in its position. The two pairs of antiparallel helices ($\alpha 1/\alpha 2$ and $\alpha 3/\alpha 5$) are nearly perpendicular to each other, and these two major parts of the structure are linked by a five-residue loop (H204–G208). As mentioned before, no amide resonances could be detected for residues H204–D206. Very likely, these residues are in conformational exchange, indicating that the $\alpha 2/\alpha 3$ loop is not entirely rigid on the millisecond timescale. Very short loops connect $\alpha 3$ to $\alpha 4$ (four residues) and $\alpha 4$ to $\alpha 5$ (three residues). Helix $\alpha 4$ is almost perpendicular to helices $\alpha 3$ and $\alpha 5$. Owing to the tight hinge-like connections with the latter two helices, helix $\alpha 4$ seems to be forced into a somewhat irregular backbone conformation.

The C-terminal part of TipAS has a large hydrophobic core. The center of this core is formed by residues F174, V175, A176, L177, M178, A189, H196, I200, H212, L215, Y219, I229, L236, Y239, M240, A243 and I244. Apparently, the strong hydrophobic contacts between these residues are responsible for the pronounced rigidity of the C-terminal part of apo TipAS despite the high lability of its N-terminus.

Analysis of ^{15}N relaxation data

The high rigidity of the folded part of apo TipAS is evident from an analysis of ^{15}N relaxation data of the backbone amide resonances (Figure 3C). Both $\{^1\text{H}\}$ - ^{15}N NOEs and ^{15}N T_1 and T_2 values (data not shown) are exceptionally uniform across this part of TipAS, ranging from D165 to H251. $\{^1\text{H}\}$ - ^{15}N NOE values close to 0.8 indicate that the internal mobility on the nanosecond timescale is very limited and comparable with other stable and well-folded proteins. In addition, significant conformational exchange on the millisecond timescale seems excluded in view of the absence of large variations in T_2 values. The uniform T_2 and T_1 values of ~ 80 ms and ~ 800 ms, respectively, are consistent with an isotropic rotational correlation time τ_c of 9 ns for apo TipAS. This value is only slightly larger (10%) than that expected for a globular protein of the mass of TipAS (16.4 kDa). The hydrodynamic drag from the unfolded N-terminus may be responsible for this minor increase in correlation time.

In contrast to the rigid C-terminal part of TipAS, much more variability in ^{15}N relaxation parameters is found for the assigned resonances in the N-terminal part (Figure 3C).

Fig. 4. Structure of the folded part of apo TipAS and chemical shift map of ligand binding. (A and B) Ribbon representations with the ligand-binding residue C214 indicated as a space-filling model. (B) is rotated relative to (A) by 225° around a vertical axis. (C) Color map of ligand-induced chemical shift changes on TipAS. Apo TipAS is shown as a space-filling model in the same orientation as (B). Residues of TipAS are color coded according to the chemical shift change of their amide resonances induced by complex formation with promethiocin A. Blue indicates weakly affected residues [$\Delta\delta_{av}(NH) < 0.3$ p.p.m.], orange indicates intermediately affected residues [$0.3 \text{ p.p.m.} \leq \Delta\delta_{av}(NH) < 0.6 \text{ p.p.m.}$] and red indicates strongly affected residues [$\Delta\delta_{av}(NH) > 0.6$ p.p.m.], where $\Delta\delta_{av}(NH) = [\Delta\delta^2(N) / 25 + \Delta\delta^2(H)]^{1/2}$. The structure of thiostrepton (Bond *et al.*, 2001) is shown close to the expected TipAS-binding site in (B) and (C). The figure was generated using the program MolMol (Koradi *et al.*, 1996).

Even at residue Q164, the $\{^1H\}$ - ^{15}N NOE value drops to 0.35, indicating large amplitude motions for this residue on the subnanosecond timescale. Although the NOEs for residues R162–Y155 increase again to values between 0.6 and 0.8, below average T_2 values for the N-terminal part of helix $\alpha 1$ indicate that this region is mobile and undergoes chemical exchange on the millisecond timescale. This mobile region continues from S154 up to the N-terminus with most of the observable residues having $\{^1H\}$ - ^{15}N NOEs lower than 0.5 and above average T_2 values. Residues missing in this analysis of the N-terminus had very weak signals, which we attribute to intermediate chemical exchange. Overall, the ^{15}N relaxation data indicate that the C-terminus of apo TipAS is well folded and stable up to the millisecond timescale, whereas the N-terminus is mostly flexible. Between these two parts of the structure, the N-terminus of helix $\alpha 1$ (K157–Q164) forms a transition region with varying degrees of mobility.

Ligand-binding studies

In order to define TipAS–antibiotic interactions, ligand-binding studies were carried out by reacting TipAS with the antibiotic promethiocin A (Figure 1). After the reaction the ligand could not be washed off, confirming that the antibiotic had bound covalently to TipAS. Figure 3B shows the central part of the ^1H - ^{15}N -HSQC spectrum recorded on the complexed TipAS protein. Most prominent is the considerably improved quality compared with apo TipAS (Figure 3A): the number of resonances in the random coil region is strongly reduced, additional signals appear and several resonances show improved lineshapes. The majority of backbone amide and $^{13}C\alpha$ and $^{13}C\beta$ resonances of the complexed protein were assigned

by the same methods as the apo protein. A comparison with the apo protein shows that the increase in resonance intensity and dispersion is mainly located at the N-terminus. The quality of most resonances in this region becomes similar to the rest of the folded protein, indicating that complex formation reduces the intermediate exchange for the N-terminus and induces the formation of a well-formed structure. This reduction of mobility of the N-terminus is also evident in the ^{15}N relaxation data. For all the assigned residues in the N-terminal region between residues N112 and Q164, the heteronuclear $\{^1H\}$ - ^{15}N NOE (Figure 3C) increases to values above 0.5, which indicate similar high backbone rigidity on the pico- to nanosecond timescale as for the C-terminal folded part of the protein. Particularly noticeable is the increase of NOE values from the apo to the complexed form of TipAS for residues in the vicinity of L114, G124 and Y145, as well as for residues S154 and Q164 (Figure 3C).

The $^{13}C\alpha$ and $^{13}C\beta$ secondary shifts for the N-terminal region of TipAS (data not shown) reveal two additional α helices, α -1 (P116–D125) and α -2 (D143–A153), that are not observed in apo TipAS (Figures 2 and 3C). Conversely, no pronounced changes of $^{13}C\alpha$ and $^{13}C\beta$ resonances are observed within the C-terminal part of TipAS. Therefore, ligand binding does not drastically alter this part of the structure. A notable exception within the C-terminal part is residue C214. The formation of the covalent bond between the dehydroalanine of promethiocin A and the sulfuryl moiety of this residue leads to strong changes for its $^{13}C\beta$ ($^{13}C\alpha$) shifts from 26.6 (63.1) p.p.m. to 36.2 (60.6) p.p.m., as well as for its backbone ^1H - ^{15}N chemical shifts (Figure 3A and B). In contrast, chemical shift changes of less than 0.4

Fig. 5. Structural similarities of TipAS and hemoglobin. (A) Structure of apo TipAS in ribbon representation with the ligand binding C214 indicated as a ball-and-stick model. The two additional N-terminal helices induced by ligand binding are indicated in green. Helix α_4 , which is not present in hemoglobin, is shown in blue. (B) Structure of *Ascaris* hemoglobin (PDB code 1ash) in ribbon representation. Helices not present in apo TipAS are shown in blue. The heme (magenta), the iron-coordinating histidine (H96) and the iron (green) and oxygen (red) atoms are shown as ball-and-stick models.

(0.1) p.p.m. are observed for the $^{13}\text{C}\alpha$ ($^{13}\text{C}\beta$) resonances of C207 upon complex formation. This is in agreement with mass spectroscopic data, which show that only C214, and not C207, forms the covalent bond with the ligand (Chiu *et al.*, 1999).

Identification of the antibiotic-binding site

TipAS has a deep linear cleft in its surface (Figure 4B and C) that is reminiscent of other ligand-binding sites in proteins (Laskowski *et al.*, 1996a). The sides of this cleft are formed by all five helices of the apo TipAS structure. The cleft is directed from the center of helix α_1 and the C-terminal end of helix α_4 towards the N-terminal end of helix α_3 , thus traversing one entire side of the molecule. The ligand-binding residue C214 is located in the center of helix α_3 at the bottom of this cleft. Its side-chain thiol group is fully solvent exposed and points in the direction of the cleft opening. In contrast, the side-chain of the non-binding C207 within loop α_2/α_3 is not pointing directly to the opening of the cleft. Owing to exchange broadening, loop α_2/α_3 is not well defined in the structure, but it is likely that C207 is partially buried by hydrophobic side chains such that it is not reachable by a reactive dehydroalanine of the antibiotic. Figure 4 also shows the wedge-like structure of thiostrepton (Anderson *et al.*, 1970; Bond *et al.*, 2001). The TipAS cleft approximately matches the size of thiostrepton such that the antibiotic would fit into this opening when its dehydroalanine tail binds to C214.

This hypothesis was confirmed by chemical shift mapping. The differences between the backbone ^1H – ^{15}N shifts of TipAS in its free and promethiocin A bound form were color coded onto the apo TipAS structure in Figure 4C. The complex formation induces chemical shift changes on almost the entire surface of the cleft. The most strongly affected residues are located at the C-terminal side of the cleft (helices α_3 – α_5) in the vicinity of C214. Therefore both the chemical shift changes and the unique location of C214 at the bottom of the cleft identify

this opening as the principal drug recognition side of TipAS. Presumably, this feature is conserved among the TipAS homologs (see below).

Similarities between heme binding by globins and antibiotic binding by TipAS

A search on the DALI server (Holm and Sander, 1993) for structures similar to apo TipAS yielded a number of weakly related proteins. The maximal z score of 4.2 is found for *Ascaris* hemoglobin (PDB code 1ash; Kloek *et al.*, 1993), which has 11% sequence identity to TipAS. Thus the stable part of apo TipAS adopts a structure that is related, albeit distantly, to the globin fold. This finding is supported by a number of other globin-related DALI hits (e.g. 1flp, 1h97, 2fal, 1ewa) with similar z scores. The relation to the globin fold becomes clearly apparent in a visual comparison of the TipAS and *Ascaris* hemoglobin structures (Figure 5).

The longer helices α_1 , α_2 , α_3 and α_5 of TipAS correspond to helices E, F, G and H in hemoglobin, whereas helix α_4 is replaced by an irregular stretch of structure. The unfolded N-terminal part of apo TipAS corresponds to N-terminal helices A–D in hemoglobin. In the absence of a complete structure for the TipAS–antibiotic complex, it is difficult to predict how the ligand-induced helices α_2 and α_1 of TipAS will relate to the hemoglobin helices. However, if these helices are in direct contact with the bound antibiotic, their positions must be similar to those of the hemoglobin helices A–D. Such a positioning is indicated by green helix symbols in Figure 5A. It is also striking that the heme pocket is very close to the expected binding site of the antibiotic. Thus one side of the heme touches helix G by a hydrophobic contact at a position (Figure 5B) corresponding to the location of the drug-binding cysteine C214 in TipAS (Figure 5A). There is a difference in the orientation of the heme and the expected orientation of the antibiotic. The chemical shift data in Figure 4C indicate that residues most strongly affected by antibiotic binding are located on

the inner side of helices $\alpha 3$ – $\alpha 5$. This suggests that the antibiotic binds into the cleft between helices $\alpha 3$ – $\alpha 5$ on one side and helices $\alpha 1$ and $\alpha 2$ on the other side. In contrast, the flat heme structure is sandwiched between helices E and F. The gap between analogous helices $\alpha 1$ and $\alpha 2$ of apo TipAS would be too small to accommodate the large body of the antibiotic in this orientation. Despite this dissimilarity, which can only be understood in detail once the structure of the TipAS–antibiotic complex is solved, other common features can be found. Thus the first macrocycle of the thiostrepton antibiotics, which contains four thiazole or oxazole rings, has some resemblance to the heme porphyrin macrocycle of four pyrrole rings. Also similar to TipAS antibiotic binding, heme binding to globins induces the folding of additional α -helices that are in contact with the ligand (Eliezer and Wright, 1996; Wakasugi *et al.*, 1997; Eliezer *et al.*, 1998).

Myoglobin (Kendrew *et al.*, 1960) and hemoglobin (Perutz *et al.*, 1960) were the first protein structures to be solved. They define the family of the heme-binding globins which share the function of oxygen transport and storage. In addition to globins, the superfamily of globin-like proteins contains the phycocyanin-like proteins (Schirmer *et al.*, 1985). The latter bind an open tetrapyrrole and are involved in the photosynthesis of cyanobacteria and red algae. In general, globin-like proteins have rather limited homology to each other and residue identities are often lower than 20% (Bashford *et al.*, 1987; Moens *et al.*, 1996). The structure of TipAS shows that the very weakly homologous C-terminal drug-recognition domains of the TipAL-like proteins also belong to the globin fold family. Thus the globin fold is used not only for binding of its classical ligands, heme and phycocyanin, but also for the recognition of a variety of antibiotics. This drug binding is linked to transcriptional regulation and drug resistance and thus is very different from the classical functions of globins.

The structural similarities raise questions about the evolution of the globin fold. It seems likely (Moens *et al.*, 1996; Wajcman and Kiger, 2002) that classical globins have evolved from unicellular organisms where their main function was to provide protection from the toxic effects of O₂, CO and NO[−]. At present, two general scenarios seem possible for the relation between the classical globins and the TipAL-like proteins: (i) the globin fold has emerged independently by convergent evolution as a motif that is especially suited for the recognition of pyrrole- or azole-containing macrocyclic structures such as heme and the thiostrepton antibiotics; or (ii) the ancestral antibiotic resistance proteins of the TipAL class have recruited heme as a ligand, thereby acquiring new functions related to protection from toxic oxygen-containing diatomic molecules.

Homology models based on the TipAS structure

The extensive sequence homology between TipAS and the C-terminal part of its homologs (see Figure 2) allowed the straightforward derivation of homology models based on the coordinates of the free TipAS structure. Using the homologous sequences and the coordinates of the best-energy TipAS structure of the simulated annealing run, models were built with the ProMod algorithm of the SwissModel server (Schwede *et al.*, 2000). Figure 6 shows

several of these models, i.e. the C-terminal domains of *B.subtilis* Mta, the *S.pneumoniae* regulator protein of PmrA and SkgA of *C.crescentus*, as well as the homologs in *S.mitis* and *B.anthraxis* together with the structure of free TipAS. Typical r.m.s.ds between the backbone C α coordinates of the models and TipAS are in the range 0.5–0.6 Å, whereas the percentage of Ramachandran core residues of the models is between 80 and 90%. It is striking how well the hydrophobic character of the antibiotic-binding cleft is conserved among the homologous proteins. For all the models, the walls of the clefts consist almost entirely of either aromatic or uncharged aliphatic residues (shown in color in Figure 6). Several strongly conserved residues can be distinguished as essential parts of the cleft wall. In particular, the hydrophobic cluster of residues L215, Y219, F225 and I229 is almost completely conserved among the homologs listed in Figure 2. Thus it is very likely that all the homologs form very similar drug-binding pockets and recognize chemically similar ligands.

The structure of TipAS as a general drug recognition motif in MDR proteins

The crystal structures of thiostrepton (Anderson *et al.*, 1970; Bond *et al.*, 2001) show that this large antibiotic is rather compact. The macrocyclic parts form a wedge-like body to which the dehydroalanine-containing tail chain is appended (Figure 4). Our data indicate that the large groove of the TipAS structure accommodates the compact body of the antibiotic. After recognition, covalent binding of the dehydroalanine in the antibiotic tail locks the antibiotic onto the protein.

Significant sequence homologies were found between TipAS and a number of other bacterial proteins. These homologies imply very similar structures and possibly similar functions for this subfamily of MerR-like proteins. In particular, the conserved amino acid composition of the modeled binding pockets suggests that chemically similar ligands are recognized by the various homologs. Currently, no such ligands have been identified for any of the other homologs. The sequence alignment in Figure 2 indicates that the homologous proteins do not contain a cysteine residue at the C214 position in TipAL. Therefore covalent binding to the ligand via this cysteine is not possible for the homologous proteins. However, covalent bond formation is not strictly necessary for *tipA* induction; thiostrepton analogs devoid of dehydroalanine can induce the expression of TipA proteins in *S.lividans*, albeit at higher concentrations than dehydroalanine-containing thiopeptides (Chiu *et al.*, 1999). For these reasons, we speculate that compounds similar to the thiostrepton ring system may be functional ligands for this class of proteins even if the possibility of covalent bond formation is not conserved.

Coupling of promiscuous antibiotic recognition, binding and folding

The TipA proteins recognize and bind not only thiostrepton, but a variety of other antibiotics such as nosiheptide, promothiocin, berninamycin, genintiocin, thioactin, thiotipin, thioxamycin and others (Chiu *et al.*, 1999). These compounds are characterized by a pyridine or reduced pyridyl moiety with one or two macrocycles containing thiazoles or oxazoles (Figure 1). Despite large

Fig. 6. Homology models based on the apo TipAS structure. Models were calculated by the SwissModel server for the following homologous sequences listed in Figure 2: *B.subtilis* (Mta), *S.pneumoniae* (the regulator of PmrA), *C.crescentus* (SkgA), *S.mitis* and *B.anthraxis*. Aromatic residues are shown in yellow, and other hydrophobic residues in red.

variations in the overall structure of the antibiotics, the first macrocycle (shaded in Figure 1) is more conserved. This surface-exposed part of the macrocycle consists of one thiazole and a second thia- or oxazole. Thus we speculate that this 'conserved' surface motif of the antibiotic structure is recognized and accommodated by the large cleft in the stable part of the apo TipAS structure (Chiu *et al.*, 1999). Upon recognition, the flexible N-terminus of apo TipAS could wrap itself around the variable parts of the antibiotic. This binding event would then stabilize the N-terminus to form additional α -helices. Many examples of such a coupled binding and folding of unstructured proteins have been revealed by NMR spectroscopy in recent years (for a review, see Dyson and Wright, 2002). As pointed out by these reviewers, the intrinsic lack of structure can confer functional advantages such as the ability to bind to different targets, perhaps in different conformations. In addition, the rate of binding and thus the efficiency of regulation by the ligand could be enhanced due to the larger 'capture radius' of an unfolded structure, a mechanism that has been proposed recently and termed 'fly-casting' (Shoemaker *et al.*, 2000). TipAS seems to provide an example where the efficient binding of various

antibiotics occurs both by the coupling of binding and folding within a flexible part of the protein and by the recognition via a preformed binding surface.

Comparison with Mta and BmrR

The only N- and C-terminal TipAL homolog characterized in some biochemical detail is the *B.subtilis* Mta protein. Unfortunately, ligands for Mta are unknown and, despite the high sequence similarity, thiostrepton binding to Mta could not be demonstrated (Baranova *et al.*, 1999). Removal of the ligand-binding domain of Mta mimics induction, as the individually expressed N-terminal DNA-binding domain of Mta (MtaN) is sufficient to induce gene transcription (Baranova *et al.*, 1999). Similarities in function and structure are expected for the N-terminal domain of TipAL. Very recently, the crystal structure of the free MtaN dimer has been determined (Godsey *et al.*, 2001). Overall, this structure resembles the DNA-binding domain of BmrR in the ternary complex with ligand and DNA (Heldwein and Brennan, 2001). However, there are minor differences in the orientation of the coiled-coil helices, which lead to a different spacing between the two

Fig. 7. Cartoon of possible *tipA*-inducing mechanism occurring upon antibiotic binding to TipAL (see text). Color coding: antibiotic, red; DNA, yellow; TipAL, green, blue, brown.

DNA-binding motifs of the MtaN dimer. In the absence of an Mta–DNA complex structure, it is difficult to assess whether this different spacing has implications for the transcription mechanism.

Recently, the structure of an N-terminal TipAL homolog, the *B.subtilis* MDR regulator protein BmrR, has been solved in a ternary complex with its cognate DNA and a drug ligand (Heldwein and Brennan, 2001). This structure has tremendously increased our understanding of MerR-like transcriptional activation. BmrR binds to DNA as a symmetric dimer in a crossed configuration such that the N-terminal DNA-recognition domain of one monomer is in direct contact with the C-terminal ligand-recognition domain of the second monomer. The structure of apo BmrR in complex with DNA is currently unknown. Based on the structure of drug-bound C-terminal part of BmrR (Zhelezнова *et al.*, 1999), it was proposed that ligand binding induces a structural change in the drug-recognition domain that transmits to the adjacent DNA-binding domain of the second monomer. The structural changes within the protein would then cause a twist of the *bmr*-promoter DNA such that the –35 and –10 regions are positioned favorably for complex formation with RNAP and subsequent initiation.

Like all MerR-like proteins BmrR has high sequence homologies to TipAL within its N-terminal DNA-binding motif and the linker region. However, their C-terminal sequences are not related and their structures are clearly different. Whereas the C-terminal drug-binding domain of TipAL forms an all-helical structure, the analogous domain of BmrR consists of a distorted eight-stranded β -barrel and two α -helices. By homology with the BmrR structure, dimerization of TipAL (Chiu *et al.*, 2001) is assumed to occur via a coiled-coil contact between the two last helices of the DNA-binding domains (Figure 2). While TipAL is dimeric in both the presence and absence of ligand, the short TipAS is always monomeric (Chiu *et al.*, 1996).

Ligand-induced helix formation as a possible mechanism for TipAL activation and *tipA* induction

It is currently unclear how binding of antibiotics to TipAL induces transcription of the *TipA* proteins. Owing to the dissimilarity of the TipAL and BmrR drug-recognition domains, the proposed activation mechanism for BmrR cannot be directly adapted to TipAL. Nevertheless, the ligand-induced partial folding of the TipAL drug-recognition domain together with general features of the drug-induced BmrR activation provide certain clues to this mechanism (Figure 7).

Our results indicate that the N-terminal third of TipAS adopts a structure that is stable on the millisecond timescale only in response to ligand binding. The binding event stabilizes the N-terminus of helix α_1 and induces the formation of the two additional helices, α_2 and α_3 . Preliminary results (data not shown) indicate a similar ligand-induced stabilization for the entire TipAL protein. This is in agreement with circular dichroism data (Chiu *et al.*, 2001) showing that the helical content of both TipAS and TipAL increases on ligand binding.

By homology with the BmrR structure, the ligand-induced helix α_2 in the C-terminal drug-binding module of TipAL is preceded by the coiled-coil helix of its N-terminal DNA-binding region. Clearly, the transition from a flexible conformation to an all-helical structure in this part of TipAL is expected to exert forces onto the DNA-binding domain. Such forces can only be transmitted efficiently if several contact sites exist between the drug-recognition domain and the DNA-binding domain. In the BmrR structure (Heldwein and Brennan, 2001), these sites are the connection via the coiled-coil helix and a second contact to the third N-terminal helix of the other monomer subunit. Thus we speculate that TipAL also forms such an additional contact. In schematic terms (Figure 7), the drug binding to TipAL causes the N-terminal part of its drug-binding domain to become rigid. The resulting forces are

Table I. Statistics of the TipAS NMR structure

R.m.s.ds from experimental distance constraints (Å)	
All (990)	0.118 ± 0.002 (0.116) ^a
Intraresidual NOEs (319)	0.147 ± 0.004 (0.145)
Sequential NOEs ($l_i - j_l = 1$) (262)	0.127 ± 0.003 (0.124)
Short-range NOEs ($1 < l_i - j_l \leq 5$) (241)	0.097 ± 0.002 (0.094)
Long-range NOEs ($l_i - j_l > 5$) (168)	0.049 ± 0.008 (0.047)
H-bonds (74) ^b	0.015 ± 0.007 (0.010)
R.m.s.ds from NMR data	
NMR quality factor Q^c	0.071 (0.070)
Residual dipolar coupling constraints (Hz) (142) ^d	0.27 ± 0.01 (0.25)
Experimental dihedral constraints (°) (167) ^e	1.10 ± 0.05 (0.91)
$^3J_{\text{HNHA}}$ coupling constants (Hz) (79)	0.25 ± 0.01 (0.39)
Deviation from idealized covalent geometry	
Bonds (Å)	0.0069 ± 0.0001 (0.0068)
Angles (°)	0.85 ± 0.01 (0.82)
Impropers (°) ^f	0.97 ± 0.02 (0.91)
ELJ (kcal/mol) ^g	−290 ± 7 (−290)
Coordinate precision (Å) ^h	
Backbone non-hydrogen atoms	0.33 ± 0.06
All non-hydrogen atoms	0.81 ± 0.07
Percentage of non-gly non-pro residues in Ramachandran regions ⁱ	
Core	92.7 (95.8)
Allowed	6.7 (4.2)
Generous	0.5 (0.0)
Disallowed	0.0 (0.0)

The statistics were obtained from a subset of the 40 best energy structures following the simulated annealing protocol with dipolar restraints incorporated by the ISAC routines (Sass *et al.*, 2001). Individual simulated annealing structures were fitted to each other using residues 165–202 and 209–251. The number of the various constraints is given in parentheses.

^aValues in parentheses correspond to data obtained from an average of the 40 structures regularized by a short simulated annealing run (300–100 K in 20 steps of 30 fs) followed by 1000-step Powell minimizations.

^bFor each backbone hydrogen bond constraint, there are two distance restraints: $r_{\text{NH}\cdots\text{O}} = 1.7\text{--}2.5$ Å; $r_{\text{N}\cdots\text{O}} = 2.3\text{--}3.5$ Å.

^cThe NMR quality factor Q is defined as the ratio of the r.m.s.d. between observed and calculated couplings and the r.m.s. of the observed couplings (Cornilescu *et al.*, 1998).

^dConsisting of 77 $^1\text{H}\text{--}^{15}\text{N}$ and 65 $^1\text{H}\text{--}^{13}\text{C}$ dipolar one-bond couplings.

^eThe dihedral angle constraints comprise 70 ϕ , 70 ψ and 27 χ_1 angles.

^fThe improper torsion restraints serve to maintain planarity and chirality.

^gELJ is the Lennard-Jones van der Waals energy calculated using the CHARMM empirical energy function and is not included in the target function for simulated annealing and restrained minimization.

^hThe coordinate precision is defined as the average r.m.s. difference between the individual simulated annealing structures and the mean coordinates. Values are reported for residues 165–202 and 209–251, i.e. for residues which do not exhibit conformational exchange or large-amplitude motions on the nanosecond timescale.

ⁱThese values were calculated using the program PROCHECK-NMR (Laskowski *et al.*, 1996b). Values are reported for the non-mobile residues^h.

then transmitted by both the coiled-coil linker and a second contact to both DNA-binding domains of the dimer. These forces result in a clamp-like movement of the TipAL dimer that leads to the observed higher affinity to the *ptipA* promoter and causes its untwisting and the subsequent recruitment of RNAP, which initiates transcription.

Materials and methods

Protein preparation

TipAS protein was produced using an *E. coli* expression system (pREP4 and pDS8 expression vectors, XL-1 Blue cells). The ^{15}N and ^{13}C labeling

was carried out by growing the bacteria on minimal medium using $^{15}\text{NH}_4\text{Cl}$ and $^{13}\text{C}_6\text{glucose}$, respectively, as the sole nitrogen and carbon sources. The protein purification procedure was slightly adapted from a method described previously (Chiu *et al.*, 1999). Further details are given in the Supplementary data, available at *The EMBO Journal* Online. The resulting TipAS protein used in this study comprises the 143 C-terminal residues of TipAL [i.e. G111–P253 (see Figure 2); this numbering system is used throughout].

Preparation of samples

Three NMR samples ($\text{U-}^{15}\text{N}$ TipAS 95% $\text{H}_2\text{O}/5\%$ D_2O ; $\text{U-}^{13}\text{C}$, $\text{U-}^{15}\text{N}$ TipAS 95% $\text{H}_2\text{O}/5\%$ D_2O ; $\text{U-}^{13}\text{C}$, $\text{U-}^{15}\text{N}$ TipAS 100% D_2O) of volume 300 μl (Shigemi NMR microtubes) were prepared as 1.5 mM protein solutions at pH 5.9, 5 mM sodium phosphate, 5 mM dithiothreitol and 0.02% NaN_3 . An additional sample ($\text{U-}^{13}\text{C}$, $\text{U-}^{15}\text{N}$ 95% $\text{H}_2\text{O}/5\%$ D_2O) of 0.8 mM TipAS was prepared containing 10 mg/ml filamentous Pf1 phages as a medium for partial orientation (Hansen *et al.*, 1998).

Preparation of the TipAS–promothiocin A complex

A reaction of TipAS with the antibiotic promothiocin A was carried out based on a procedure described previously (Chiu *et al.*, 1996). The reaction mixture was washed to remove excess antibiotic and dimethylsulfoxide, and the buffer was exchanged for 5 mM potassium phosphate pH 5.9 in Centricon concentrators (Millipore). NMR samples were prepared as described above.

NMR spectroscopy

A set of standard triple- and double-resonance assignment, quantitative J coupling, ^{15}N -relaxation and NOESY experiments similar to those described (Grzesiek *et al.*, 1997) were performed at 25°C on Bruker DRX 600 and 800 MHz spectrometers. Standard data processing and analysis was carried out as described (Grzesiek *et al.*, 1997).

Structure calculation

NOE distance, torsion angle and residual dipolar restraints derived from the NMR data are listed in Table I. Structure calculations were carried out with a simulated annealing protocol using a modified version of the program X-PLOR (Brünger, 1992) as described (Grzesiek *et al.*, 1997). The structural statistics for the best 40 structures are given in Table I. The coordinates and structural restraints have been deposited in the Brookhaven Protein Data Bank under accession code 1NY9.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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