PROTEIN STRUCTURE REPORT

NMR structure of the calflagin Tb24 flagellar calcium binding protein of *Trypanosoma brucei*

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Abstract: Flagellar calcium binding proteins are expressed in a variety of trypanosomes and are potential drug targets for Chagas disease and African sleeping sickness. The flagellar calcium binding protein calflagin of *Trypanosoma brucei* (called Tb24) is a myristoylated and palmitoylated EF-hand protein that is targeted to the inner leaflet of the flagellar membrane. The Tb24 protein may also interact with proteins on the membrane surface that may be different from those bound to flagellar calcium binding proteins (FCaBPs) in *T. cruzi*. We report here the NMR structure of Tb24 that contains four EF-hand motifs bundled in a compact arrangement, similar to the overall fold of *T. cruzi* FCaBP (RMSD = 1.0 Å). A cluster of basic residues (K22, K25, K31, R36, and R38) located on a surface near the N-terminal myristoyl group may be important for membrane binding. Non-conserved residues on the surface of a hydrophobic groove formed by EF2 (P91, Q95, D103, and V108) and EF4 (C194, T198, K199, Q202, and V203) may serve as a target protein binding site and could have implications for membrane target recognition.

Keywords: FCaBP; *Trypanosoma brucei*; calcium; EF-hand; myristoyl switch; NMR

Abbreviations: HSQC, heteronuclear single quantum correlation; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RMSD, root mean square deviation; Tb24: *T. brucei* flagellar calcium binding protein; calcium binding protein 1.

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Introduction

Flagellar calcium-binding proteins (FCaBPs) are immunogenic proteins found in the flagellum of protozoan parasites: *Trypanosoma cruzi*,¹ *Trypanosoma brucei*,² and *Trypanosoma rangeli*³ (see Fig. 1). FCaBPs contain four EF-hand calcium-binding motifs^{5,6} (Fig. 1), the third and fourth (EF-3 and EF-4) of which bind calcium.⁷ The protein is modified at the N terminus by covalent attachment of myristate at Gly2 and palmitate at Cys4, both of which along with conserved lysine residues near the N-terminus⁸ are required for association with the inner leaflet of the flagellar membrane.⁹ Calcium is required for stable flagellar localization as well, since *T. cruzi*

Figure 1. Alignment of the primary sequence of *T. brucei* calflagin Tb24 with those of *T. rangeli* FCaBP and *T. cruzi* FCaBP. Secondary structural elements indicated schematically were derived from analysis of NMR data ($^3J_{\text{HNH}\alpha}$, chemical shift index⁴ and sequential NOE patterns). The four EF-hands (EF1, EF2, EF3, and EF4) are highlighted green, salmon, cyan, and yellow, respectively. Residues in the 12-residue Ca^{2+} -binding loops are underlined. Invariant basic residues implicated in membrane binding are colored blue. Non-conserved residues in the hydrophobic groove are highlighted in bold. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FCaBP can be washed out of detergent-permeabilized trypanosomes if calcium chelators are included in the wash solutions. The N-terminal acylation and calcium-dependent membrane localization of *T. cruzi* FCaBP suggested that the protein may possess a functional calcium-acyl switch, similar to the Ca^{2+} -myristoyl switch observed previously for recoverin^{10,11} and other members of the neuronal calcium sensor (NCS) family.¹²

Three FCaBP homologs in *T. brucei* have been characterized and named the calflagins: Tb24, Tb17, and Tb44.¹³ The calflagins are similar in sequence to *T. cruzi* FCaBP (Fig. 1) and exhibit Ca^{2+} -induced localization to the flagellar membrane during cell motility, chemotaxis, and cell signaling. *T. brucei* calflagin Tb24 possesses different membrane binding properties compared to that of *T. cruzi* FCaBP. Tb24 requires both myristoylation and palmitoylation for binding to flagellar membranes,^{14,15} whereas the unpalmitoylated Tb24 is trafficked to the pellicular (cell body) membrane.¹⁶ This is in stark contrast to *T. cruzi* FCaBP, whose unpalmitoylated form is mislocalized in the cytoplasm.⁹ The different membrane targeting properties of modified forms of these similar proteins is perhaps due to some protein struc-

tural difference between the two, or due to different lipid composition of various trypanosome membrane domains.¹⁷ *T. brucei* Tb24 and *T. cruzi* FCaBP may also differ by interacting with distinct membrane-bound protein targets.^{18,19}

Atomic resolution structures of *T. brucei* FCaBPs are needed to elucidate their Ca^{2+} -induced conformational changes that control membrane-targeting. We report here the NMR structure for Ca^{2+} -free calflagin Tb24 (henceforth referred to simply as Tb24).

Results

NMR spectroscopy of Tb24

^{15}N - ^1H heteronuclear single quantum correlation (HSQC) NMR spectra of unmyristoylated and unpalmitoylated Tb24 in the Ca^{2+} -free state exhibit the expected number of backbone amide NMR peaks, indicating that the protein is monomeric and adopts a stable three-dimensional fold.²⁰ N-terminal myristoylation and/or palmitoylation of Tb24 causes the protein to aggregate under NMR conditions due to a solvent exposed N-terminus (see below), and therefore the myristoylated and/or palmitoylated forms of

Table I. Structural Statistics for the Ensemble of 20 Calculated Structures of Tb24

NOE restraints (total)	2446
Intra ($ i - j = 0$)	650
Medium ($1 \leq i - j \leq 4$)	1037
Long ($ i - j > 4$)	759
Dihedral angle restraints (ϕ and Ψ)	238
Hydrogen bond restraints in α -helical regions	156
RMSD from ideal geometry	
Bond length (Å)	0.0061 ± 0.00012
Bond angles (°)	1.96 ± 0.0013
Ramachandran plot	
Most favored region	79.1%
Allowed regions	20.9%
Disallowed regions	0%
RMSD of atom position from average structure	
β -sheet and α -helical regions (main chain atoms)	0.70 ± 0.09 Å
β -sheet and α -helical regions (non-hydrogen atoms)	1.25 ± 0.09 Å

Tb24 were not examined in this study. The first 26 residues from the N-terminus exhibited random-coil amide chemical shifts with non-uniform intensities, indicating that the N-terminal region is indeed solvent-exposed and dynamically disordered. C-terminal residues (206–218) also have chemical shifts consistent with a random coil. The remaining residues (27–205) exhibited highly dispersed amide chemical shifts with uniform intensities indicative of a folded core domain.

NMR-derived structure of Tb24

NMR assignments for Ca^{2+} -free Tb24 were described previously²⁰ (BMRB no 18011). These assignments served as a basis for obtaining nuclear Overhauser effect (NOE) distances, hydrogen-bonds, and dihedral angle restraints from the NMR data used as input for calculating the atomic-resolution structure by restrained molecular dynamics simulations as described in the Methods section. Table I summarizes the structure statistics for the 20 lowest energy conformers (Protein Databank accession no. 2lvv). The calculated structures were validated using PROCHECK, which shows that 79.1% of the residues belong to the most favorable region in the Ramachandran plot.

The final NMR-derived structures of Ca^{2+} -free Tb24 are illustrated in Figure 2 and summarized in Table I. The 20 lowest energy conformers when superimposed have an overall main chain root mean square deviation (RMSD) of 0.7 Å (Supporting Information Fig. S1). The Tb24 main chain structure contains a total of eight α -helices and four β -strands: $\alpha 1$ (residues 26–38), $\alpha 2$ (residues 47–60), $\alpha 3$ (residues 70–79), $\alpha 4$ (residues 90–105), $\alpha 5$ (residues 119–139), $\alpha 6$ (residues 152–164), $\alpha 7$ (residues 172–182), $\alpha 8$ (resi-

dues 189–204), $\beta 1$ (residues 67–69), $\beta 2$ (residues 116–118), $\beta 3$ (residues 149–151), and $\beta 4$ (residues 186–188). Tb24 contains four EF hands: EF1 (green residues 22–55), EF2 (red, residues 61–91), EF3 (cyan residues 101–130), and EF4 (yellow, residues 140–167). The four EF-hands associate into two pairs through a two-stranded β -sheet arrangement: EF1 (green) pairs with EF2 (red), while EF3 (cyan) pairs with EF4 (yellow). The two pairs of EF-hands are connected by a long, central helix ($\alpha 5$) formed by merging the exiting helix of EF2 into the entering helix of EF3. This topology causes three long, central helices ($\alpha 4$, $\alpha 5$, and $\alpha 8$) to interact closely as a twisted, vertical bundle, causing contact between EF2 and EF4. The overall main chain structure of Tb24 is very similar to that of *T. cruzi* FCaBP (RMSD is 1.0 Å when comparing main chain atoms). An important structural difference between Tb24 and FCaBP can be seen in the exposed groove region [see dashed curve in Fig. 2(A)] that is lined by hydrophobic residues (V108, V175, C194, and T198) that are not conserved in FCaBP.

Surface properties of Tb24

A space-filling representation of Tb24 reveals a cluster of positively charged side-chain atoms from Arg and Lys residues on one side of the protein surface [blue in Fig. 2(B)] with an exposed hydrophobic patch on the opposite side [yellow in Fig. 2(C)]. We propose that the charged protein surface in Figure 2(B) may make contact with flagellar membranes. Indeed, N-terminal Arg and Lys residues (K25, K31, R36, and R38) shown in Figure 2(B) are conserved in all FCaBPs (highlighted blue in Fig. 1) and have been implicated in membrane binding via electrostatic interactions with negatively charged lipid head groups on the flagellar membrane.¹⁹ Exposed hydrophobic residues in the N-terminal helix (A28, V32, and C39) are not conserved and might help explain the distinct membrane binding properties between Tb24 and *T. cruzi* FCaBP.

The protein surface on the opposite face [Figure 2(C)] forms a groove that contains exposed hydrophobic residues from EF2 (L104 and V108) and EF4 (A173, A174, L175, L179, C194, W195, T198, L201, and V203). Many exposed residues in the groove (P91, Q95, D103, C194, T198, K199, Q202, and V203) are not conserved in FCaBP and may play a role in controlling target specificity. We propose that Ca^{2+} -induced conformational changes in EF4 might alter the arrangement of the groove residues, and these residues likely represent a binding site for the Ca^{2+} -induced interaction with membrane-bound protein targets.

Ca^{2+} -free Tb24 is a monomer

Previous studies suggested that flagellar calcium binding proteins in *T. cruzi* may form functional

Figure 2. NMR-derived structure of Tb24. The average main chain structure is shown by a ribbon diagram (A) and space-filling representations (B and C). N-terminal residues (1–26) are unstructured and not shown. In panel (A), EF-hands are colored as defined in Figure 1 and the exposed hydrophobic groove is marked by a dashed curved. In panels (B,C), acidic residues (Asp and Glu), basic residues (Arg, His, Lys), and hydrophobic residues (Ile, Leu, Phe, Met, Val) are colored red, blue, and yellow, respectively. The surface in panel (B) is proposed to interact with the flagellar membrane, whereas the surface in panel (C) (hydrophobic groove) is postulated to bind to protein targets. Protein databank accession no. 2lvv.

dimers.^{18,19} ¹⁵N-NMR relaxation analysis (R_1 and R_2) of Tb24 indicates an average rotational correlation time of ~ 12 ns, suggesting that Tb24 forms a monomer under NMR conditions. Also, we did not observe any significant chemical shift changes in NMR spectra recorded as a function of protein concentration ($50 \mu\text{M}$ – 1 mM) and did not observe any intermolecular NOEs. Thus, Ca^{2+} -free Tb24 appears to be a monomer under NMR conditions.

Discussion

The NMR structure of Tb24 (Fig. 2) has implications for its Ca^{2+} -induced membrane-targeting mechanism. The unstructured N-terminal region (residues 1–25) that is myristoylated at G2 and palmitoylated at C3 is solvent exposed and would explain how the attached fatty acyl groups might serve as membrane anchors by inserting inside the lipid bilayer. Exposed basic residues in the N-terminal region [highlighted blue in Fig. 2(B)] are suggested to assist in membrane binding by interacting with negatively charged head-groups on the membrane surface. A similar combina-

tion of electrostatic effects and myristoylation was seen previously for membrane binding by the myristoyl-electrostatic switch of Src and MARCKS protein.²¹ Lastly, non-conserved residues in the hydrophobic groove region [highlighted yellow in Fig. 2(C)] may represent a binding site for protein targets¹⁴ and perhaps explain the different membrane-targeting properties of Tb24 versus *T. cruzi* FCaBP.^{9,14,16}

Methods and Methods

Expression and purification of Tb24

Recombinant and uniformly ¹⁵N- or ¹⁵N/¹³C-labeled unmyristoylated Tb24 was expressed in *Escherichia coli* strain, BL21(DE3) grown on M9 medium supplemented with ¹⁵N-NH₄Cl and/or ¹³C₆-glucose. Recombinant protein expression was induced by exogenously adding 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) to cells grown overnight at 25°C . Typically, a 1-L culture yields about 20 mg of purified protein. Detailed procedures for purifying Tb24 are described elsewhere.¹⁴

NMR spectroscopy

Samples of recombinant Ca^{2+} -free Tb24 (0.7 mM) were prepared in 90%/10% $\text{H}_2\text{O}/\text{D}_2\text{O}$ or 100% D_2O with 10 mM sodium phosphate (pH 7.0), 4 mM dithiothreitol (DTT)- d_{11} and 0.3 mM ethylene diamine tetra acetic acid (EDTA)- d_{12} . NMR experiments were conducted using Bruker Advance 600 MHz spectrometer equipped with a triple resonance cryogenic probe. All experiments were performed at 298 K. Backbone and side-chain chemical shift assignments were obtained using ^{15}N -HSQC, HNCO, HNCACB, CBCACONH, HBHACONH, and ^{15}N -HSQC-TOCSY (mixing time of 60 ms) spectra.²² Methyl group side-chain resonances were assigned using ^{13}C -CT-HSQC and ^{13}C -HCCH-TOCSY. For aromatic side-chain chemical shift assignments, HBCBCGCDHD, HBCBCGCDCEHE, ^{13}C -CT-HSQC-TOCSY spectra²³ along with ^{13}C -HSQC-NOESY, recorded with a mixing time of 120 ms, were used. NMR data were processed using NMRPipe²⁴ software package and analyzed using SPARKY.

Structure calculation

Structures of Tb24 were calculated by Xplor-NIH 2.23²⁵ using a simulated annealing protocol. Dihedral angle restraints derived from TALOS,²⁶ NOE distance restraints, and hydrogen bond distance restraints were employed to calculate ensemble model structures using the YASAP protocol.²⁷ After refinement, the 10 lowest energy structures (from a total of 100) were selected and analyzed. The NMR-derived structures of Tb24 were assessed by PROCHECK. The NMR-derived ensemble structures of Tb24 were deposited in the Protein Data Bank, with accession code 2lvv.

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