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TWO MOTIFS UNIQUE TO TRYPANOSOME TRANSCRIPTION FACTOR IIB ARE INTRINSICALLY DISORDERED

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Abstract- The structural characterization of trypanosome proteins can reveal significant differences relative to their human homologs which may be exploited as novel therapeutic targets. The recent determination of the C-terminal domain of the *T. brucei* TFIIB transcription factor (tTFIIBc) revealed that its DNA binding surface is much poorer in positive charges compared to the human protein. The *T. brucei* protein also contains an 18 residue internal segment and a 32 residue C-terminal extension which are not found in human TFIIBc, but their missing electron density barred the determination of their conformations by X-ray crystallography. Here, we have characterized structurally peptides corresponding to these motifs using circular dichroism, fluorescence and nuclear magnetic resonance spectroscopies. The internal segment was found to be mostly disordered, with a slight tendency to adopt helical conformations as evidenced by a modest induction of helicity by 40% TFE and several weak $^{1}HN_{i}$-$^{1}HN_{i+1}$ NOES. The peptide corresponding to the C-terminal extension is essentially disordered. These results show that the lack of electron density was not due to crystallization conditions or packing but to intrinsic disorder. Considering that intrinsically disordered motifs frequently mediate protein-protein interactions, these results are consistent with our proposal that the internal segment and C-terminal extension may compensate for the low positive charge density of *T. brucei* TFIIB by recruiting auxiliary transcription factors.
Keywords- *Trypanosoma brucei*, Sleeping Sickness, NMR Spectroscopy, Intrinsically Disordered Proteins, Circular Dichroism, Fluorescence Spectroscopy.

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Introduction

Sleeping sickness is caused by *Trypanosoma brucei*. When untreated, it produces irreversible neurological damage and is invariably fatal, claiming some 50000 victims per year. Effective treatments are available but they must be administered by injection and have serious side effects including death in a small percentage of cases [1]. By specifically targeting required proteins, it should be possible to develop more selective therapies with fewer side effects and which could be given orally.

The transcription factor IIB (TFIIB) plays crucial roles in RNA transition initiation. It binds DNA, RNA polymerase II, the TATA box binding protein and organizes other transcription initiation factors [2] [3]. Trypanosome TFIIB is a potentially good therapeutic target because it is an essential protein which is distinct from its human counterpart on the level of sequence and tertiary structure; in particular, two striking differences have been recently described for the human and trypanosomal TFIIB protein [4]. First, human TFIIB is much richer in positively charged residues which are strategically positioned to interact with DNA. Second, *T. brucei* TFIIB has an additional helix, a novel 3/10 helix (H3'A)+loop segment and a 32 residue C-terminal extension. These structures are conserved in *Trypanosoma cruzi,* the pathogen responsible for Chagas disease, but not in the human protein. The loop segment and the C-terminal extension do not show electron density, either because: 1) the conditions of the crystallization experiment or the packing constraints within the crystal lattice prevent them from adopting a well ordered conformation, or 2) these motifs are intrinsically disordered.

Here, we test between these two possibilities by characterizing structurally peptides corresponding to the helix H3'A + loop and C

-terminal segments which are not observed in the X-ray structure using CD, Fluorescence and NMR spectroscopies. We show that both peptides are largely disordered, although the peptide corresponding to the helix H3'A + loop has a slight tendency to adopt helical conformations. Therefore, the absence of electron density for these segments in the tTFIIBc crystal structure is due to a lack of structure and is not a consequence of the crystallization conditions or tTFIIBc packing within the crystal lattice. These results are consistent with our previous proposal that these novel elements may compensate for the lower positive charge density of trypanosomal TFIIB by recruiting additional factors [4].

Materials and Methods

Synthetic Peptides- Two synthetic peptides were acquired from Genescript Corp. One peptide had the sequence: QTLSW EQGTH ISKGK ESDL corresponding to the short 3/10 helix (residues QTLSW) and the following 14 residues which lack electron density in the X-ray structure, except the Cys was substituted by Ser (underlined). The second peptide had the sequence: DSGEP TAGKR KVDKN SEPEA SGSTK RVKRE ET of the 32 residues at the C-terminus of tTFIIBc which are invisible in the X-ray structure [4]. The Cys present in this second peptide was also substituted by Ser (underlined) to avoid potential problems with cysteine oxidation or disulfide bond formation. These peptides are called the "loop" and "C-terminal" peptides, and correspond to residues 256-274 and 314-345 in the full length tTFIIB protein, respectively. Both peptides were over 95% pure as judged by HPLC and their identities were confirmed by mass spectrometry and NMR spectroscopy.

Bioinformatics- Putative folded and intrinsically disordered segments in the amino acid residue sequence of the C-domains of TFIIB from *T. brucei* and *T. cruzi* were predicted using the program FoldIndex© (http://bioportal.weizmann.ac.il/fldbin/findex, [5]. The Kyte and Doolittle hydrophobicity scale [6] and a residue window size of ten were used.

CD spectroscopy- A Jasco 810 spectropolorimeter was employed for CD spectroscopy. Spectra were recorded over a wavelength range of 190 - 260 nm using 0.5 nm steps, a one nm slit-width, and a scan speed of 20 nm·min^{-1}. Four scans were acquired and averaged per spectrum. The samples contained 100 mM KCl, 10 mM K_2HPO_4, 10 mM HAc and 127 μM (loop peptide) or 91 μM (C-terminal peptide). Some spectra also were recorded on samples in 40% TFE, 100 mM KCl, 10 mM KH_2PO_4 and 10 mM HAc at pH 7. Spectra were obtained at pH 3.5 and 7.0 and at 10.0 and 37.0 ºC. Reference spectra on this buffer alone or buffer with 40% TFE were recorded and subtracted from the spectra recorded on peptide samples.

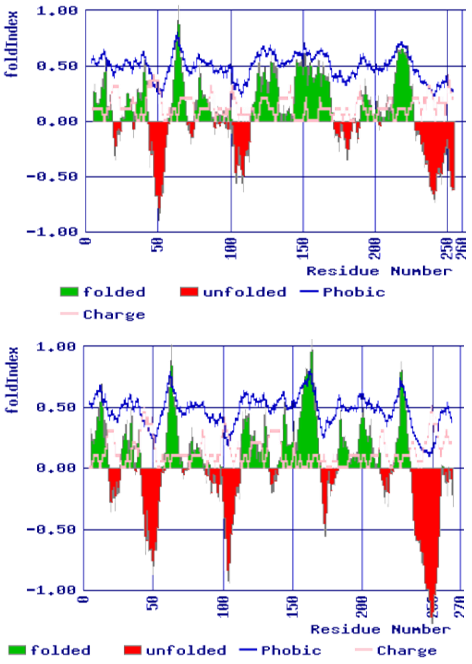
Fluorescence spectroscopy- A Jobin-Yvon Fluoromax 4 instrument was used to obtain fluorescence spectra. The intrinsic fluorescence spectrum of the loop peptide, whose concentration was 61 μM, was measured at 20 ºC in 10 mM K_2HPO_4, 10 mM HAc buffer, pH 7.0. The excitation wavelength was 280 nm and emission was recorded from 295 to 400 nm. This spectrum and those recorded with ANS were recorded in 1 nm steps scanning at 0.4 s per nm and the slits for excitation and emission were both 2 nm. The excitation and emission wavelengths were calibrated using the Xe lamp excitation maximum and the Raman emission line of water.

ANS fluorescence- ANS, whose concentration was 120 μM, was excited at 365 nm and its emission was recorded over 400 - 650 nm. Spectra were recorded at 20 ºC in buffer at pH 7.0 alone or in the presence of 61 μM loop peptide or 52 μM C-terminal peptide.

NMR Spectroscopy- All NMR spectra were acquired on a Bruker AV 800 NMR spectrometer equipped with a cryoprobe. Samples contained peptides at 0.3 to 4.5 mM concentration, 100 mM KCl, 5 mM HAc (d_3), 5 mM KH_2PO_4, 1 mM NaN_3. Samples also contained 50 mM sodium 2,2 dimethyl-2-silapentane sulfonate as the internal ^{1}H chemical shift reference. This signal was also used to indirectly reference the ^{13}C chemical shifts [7]. Samples were dissolved in 9:1::H_2O:D_2O for recording 1D ^{1}H and 2D ^{1}H TOCSY (60 ms mixing time), NOESY (150 ms mixing time), and ^{1}H-^{15}N HSQC spectra or 99.9% D_2O for recording 2D ^{1}H-^{13}C HSQC spectra. The HSQC spectra were recorded using the natural abundance of ^{13}C and ^{15}N. The pH of the samples was 5.30 to 5.40 except for the initial series of 1D ^{1}H spectra described below. A WATERGATE module was used for solvent suppression for the spectra in 90% H_2O and presaturation was employed for samples containing 99.9 % D_2O. All spectra were recorded at 10 ºC. Spectra were transformed and processed using the Bruker TOPSPIN program and were assigned with the aid of the program SPARKY [8].

Results

Bioinformatics- The amino acid residue composition of the loop segment and C-terminal extension are rich in polar and charged residues and rather poor in larger hydrophobic residues. Such a composition is typical of intrinsically disordered segments (Liu et al. Biochemistry 2006 45:22 6873). Both segments, particularly the C-terminal extension, are predicted to be intrinsically disordered by a sequence-based algorithm FoldIndex© (Supporting Figure 1). Interestingly, similar results are obtained for the TFIIB sequence of *T. cruzi*.



Supporting Fig.1-Bioinformatics Analysis of the *T. brucei* (top panel) and *T. cruxi* TFIIB C-terminal domain sequences using FoldIndex©.

CD spectroscopy: The secondary structure in the loop and C-terminal peptides was measured by CD spectroscopy. Spectra were measured both in buffer as well as buffered 40% TFE, a co-solvent which is known to enhance helix formation in protein segments possessing an intrinsic propensity to adopt helical conformations [9-11]. The results are shown in Figure (1). In aqueous solution, both the loop and C-terminal peptides show features typical of an ensemble of random coil conformations; namely, values close to zero at 222 nm and minima near 200 nm. In the presence of 40% TFE, modest (10 ºC) and slight (37ºC) induction of helical structure is observed in loop peptide's spectra (Figure 1 A and B), whereas smaller changes are seen in the C-terminal peptide's spectra (Figure 1C and D).

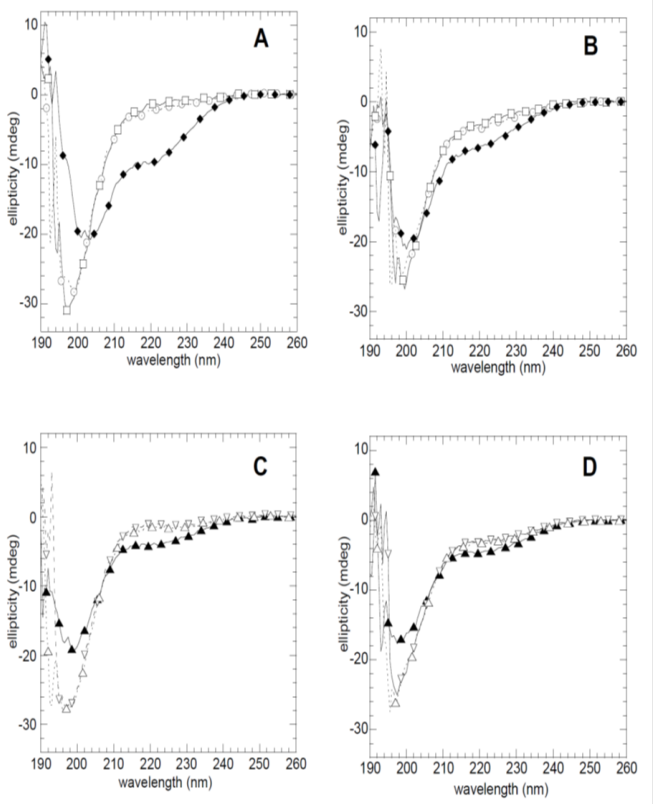


Fig. 1- CD Spectra of tTFIIB Peptides
A. Loop peptide, 10 ºC. open circles and solid line, pH 3.5; open squares and dotted line, pH 7.1; filled diamonds and solid line, pH 7.1 with 40% TFE. **B**. Loop peptide, 37 ºC. the symbols have the same meaning as panel A. **C**. C-terminal peptide, 10 ºC. open triangles and solid line, pH 3.5; inverted open triangles and dotted line, pH 6.8; filled triangles and solid line, pH 6.8 with 40% TFE. **D**. C-terminal peptide, 37 ºC. the symbols have the same meaning as panel C**.**

Fluorescence spectroscopy- The fluorescence spectrum of the loop peptide reveals an emission maximum above 350 nm, which is consistent with the Trp indole group being solvent exposed and not buried (Figure 2A). The loop and C-terminal peptides do not enhance the fluorescence of ANS (Figure 2B). This suggests that they have no partly ordered, solvent exposed hydrophobic patches [12].

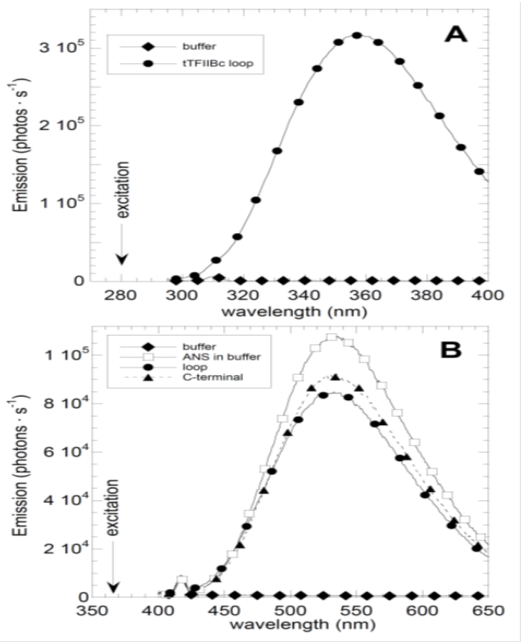
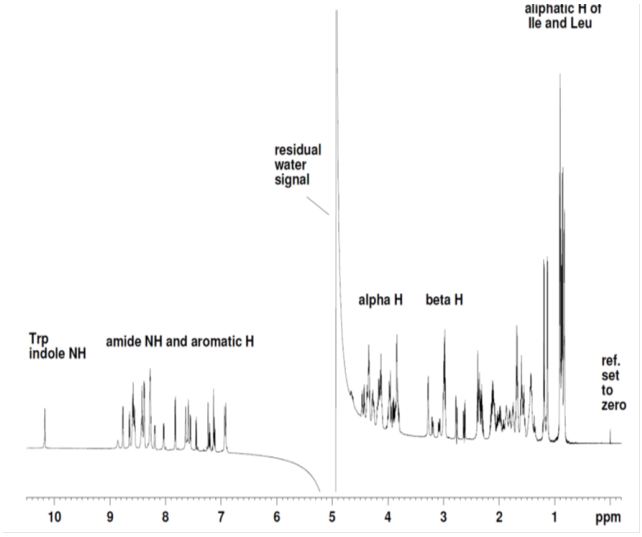


Fig. 2- Intrinsic and ANS Fluorescence Spectra of tTFIIB peptides **A.** Fluorescence spectrum of the loop peptide recorded at pH 7.0, 20 ºC. **B**. Fluorescence spectra of ANS alone in buffer or in the presence of the tTFIIB peptides recorded at pH 7.0, 20ºC.

1D ^{1}H NMR Spectroscopy- The peptides were dissolved in one volume of milliQ H_2O, then one volume of 2x buffer. The peptides dissolved cleanly with no sign of turbidity. An initial series of 1D ^{1}H spectra were recorded for each peptide at low concentration (0.3 to 0.5 mM) at pH 3, 5, 7 and 10. Sharp lines and low dispersion characteristic of unfolded peptides were observed (Supporting Figure 2). At alkaline pH, the ^{1}HN signals disappear within 30 minutes due to rapid exchange of these protons with solvent (data not shown). This suggests that these peptides lack stable intramolecular hydrogen bonds and therefore that they lack stable helices and beta sheets.



Supporting Fig. 2- 1D ^{1}H NMR Spectrum of the tTFIIBc loop peptide in 100 mM KCl, 5 mM K_2HPO_4, 5mM HAc, 3 mM NaN_3, pH 5.3, 90% H_2O, 10% D_2O at 10ºC.

2D NMR Spectroscopy

The 2D ^{1}H TOSCY and NOESY spectra of the loop and C-terminal peptides were assigned by standard strategies [13]. These assignments were corroborated and, in the case of some of the longer side chains, completed by analysis of the ^{1}H-^{13}C HSQC and ^{1}H-^{15}N HSQC (C-terminal peptide only) spectra (Figure 3). The assignments for the loop and C-terminal peptides are listed in Tables 1 and 2, respectively. No significantly upfield shifted peaks, which generally arise from the packing of aliphatic groups on aromatic rings, were observed. All the ^{1}HN signals occupy a narrow range of chemical shift values at 10 ºC (7.9 - 9.0 ppm). These observations are hallmarks of peptides lacking preferred conformations.

Spectra were recorded on 1 - 4 mM peptides samples in 100 mM KCl, 5 mM K_2HPO_4, 5 mM HAc, 1 mM NaN_3 buffer, pH 5.3 at 10 ºC in 90% H_2O, 10% D_2O, except B, which was recorded in 99.9 % D_2O buffer.

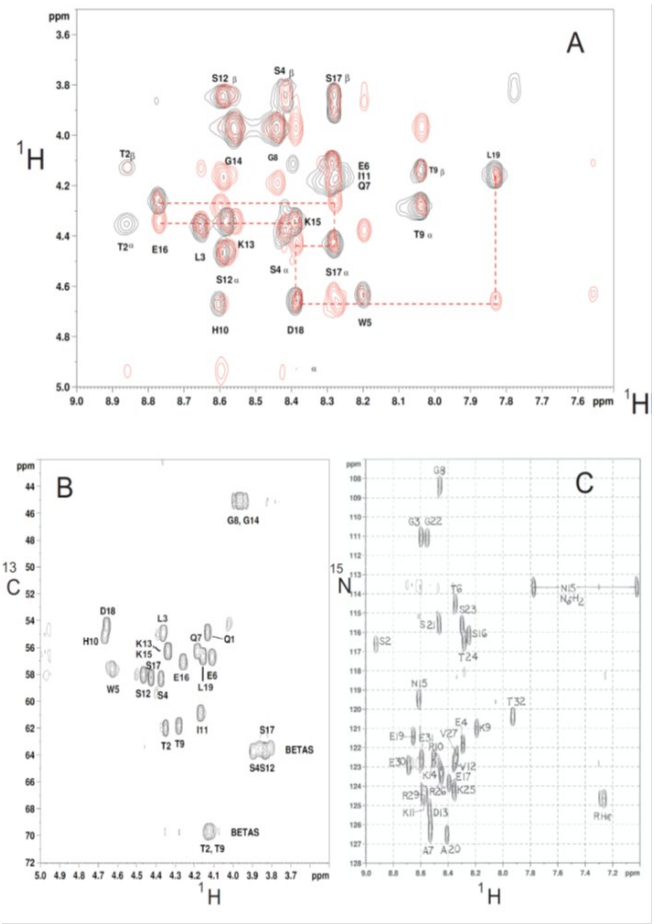


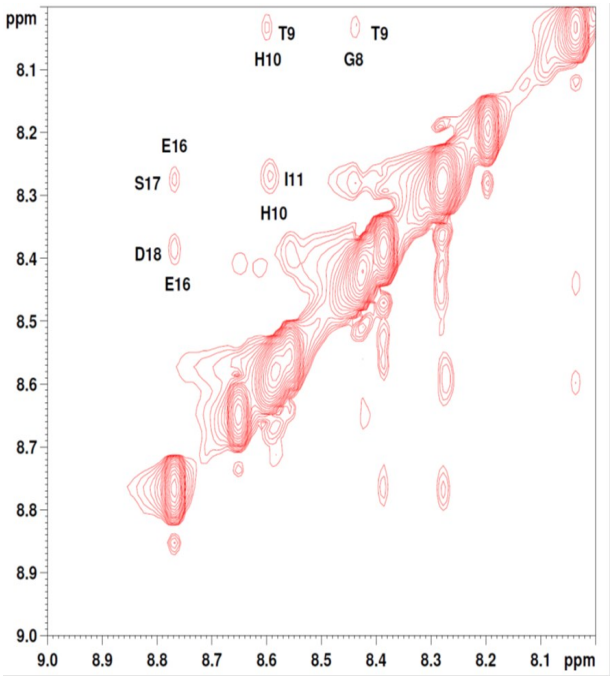
Fig. 3- Assignment of the NMR spectra of tTFIIBc peptides

A. Regions of the 2D ^{1}H TOCSY (black) and NOESY (red) spectra of the loop peptide. The ^{1}HN-$^{1}H\alpha$ crosspeaks are labeled. The broken red line shows the sequential connectivities of residues K15 to L19 via NOE signals.

B. $^{13}C\alpha$-$^{1}H\alpha$ region of the HSQC spectrum of the loop peptide. The Ser and Thr $^{13}C\beta$-$^{1}H\beta$ resonances are also labeled.

C. The ^{15}N-^{1}H HSQC spectrum of the C-terminal peptide, showing the assigned signals. The Arg side chain peak at 7.27 ppm (^{1}H) is folded in the ^{15}N dimension.

The NOESY spectrum recorded on the loop peptide shows some signs of minor populations of ordered conformations; namely, there are two weak crosspeaks arising from the Trp and a Leu or Ile side chain (data not shown). These signals likely arise from medium range contacts between W5 and L3. There are several HNi - HNi+1 NOE crosspeaks arising from residues G8, T9, H10 and I11; and E16, S17 and D18 of the loop peptide (Supporting Figure 3). Such signals are characteristic of turn or helical structure. This turn or helical structure is not significantly stable since as previously noted, the NH readily exchange at higher pH values. Additional HNi - HNi+1 NOE crosspeaks from W5, E6 and Q7; and S12, K13 and G14 might be present but can not be observed due to their proximity to the diagonal. No such signs of even minor populations of ordered structure are seen in the C-terminal peptide.



Supporting Fig. 3- 2D ^{1}H NOESY NMR Spectrum of the tTFIIBc loop peptide in 100 mM KCl, 5 mM K_2HPO_4, 5mM HAc, 3 mM NaN_3, pH 5.30% H_2O, 10% D_2O at 10ºC. $^{1}HN_i$-$^{1}HN_{i+1}$ NOE crosspeaks indicative of tight turn or helical structure are labeled.

The population of partial secondary structure was assessed on the level of individual residues by comparing the experimental $^{1}H\alpha$ and $^{13}C\alpha$ values with reference "random coil" values [14]. Conformation chemical shift ($\Delta\delta = \delta$ exp – δ r.c.) values of -0.40 ppm for $^{1}H\alpha$ and +3.10 ppm for $^{13}C\alpha$ are expected for 100% helical structures, and values of -0.10 ppm for $^{1}H\alpha$ and +0.70 ppm for $^{13}C\alpha$ are commonly used as thresholds for identifying partial structure formation. Similar $\Delta\delta$ values but with opposite signs are used for detecting and quantifying β-structures. Except for the terminal residues, whose chemical shift values are altered by end charge effects, the $\Delta\delta$ values are small and below the thresholds for detecting structure in the C-terminal peptide (Figure 4). For the loop peptide, a weak tendency towards helical conformations was detected for residues E6 and Q7.

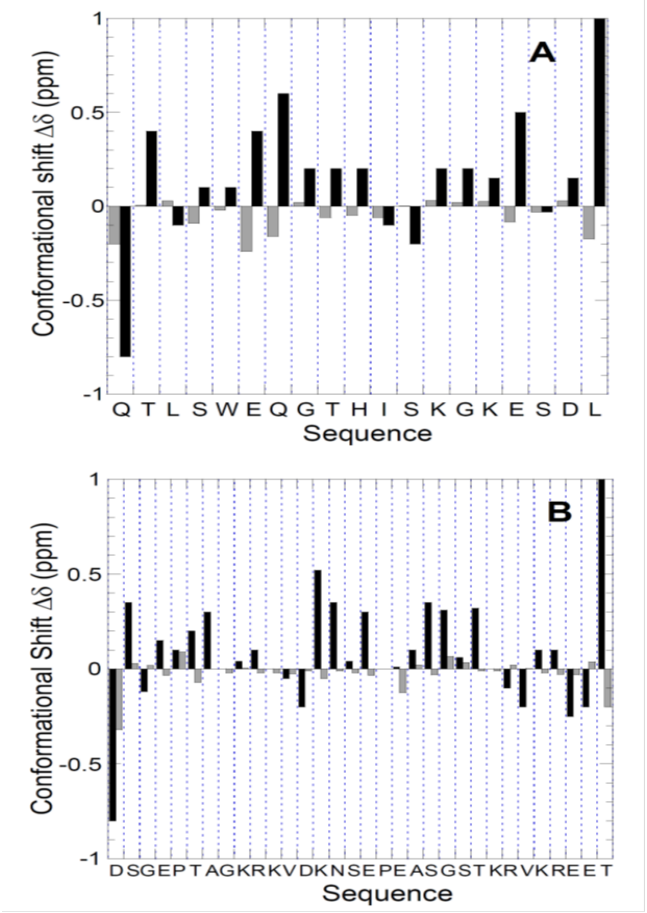


Fig. 4- Backbone Conformational Shifts for the loop (A) and C-terminal peptides (B) at 10 ºC in pH 5.3 buffer with 100 mM KCl. $\Delta\delta$ values for $^{1}H\alpha$ (gray bars) and $^{13}C\alpha$ (black bars) are shown.

Discussion

Taking into account all the data, we conclude that the C-terminal peptide does not have a preferred conformation. Therefore, the absence of electron density for the C-terminal segment in tTFIIBc is due to a lack of structure and is not a consequence of the conditions of the crystallization experiments or packing of tTFIIBc within the crystal lattice in such a way that impedes the correct folding of these segments. The loop peptide is also largely disordered but has a slight tendency to adopt helical conformations. Interestingly, the N-terminal region of this peptide (residues Q1-T2-L3-S4-W5; corresponding to Q256-T257-L258-S259-W260, in the full-length protein) adopts a short 3/10 helix in the crystal structure of tTFIIBc. This strongly suggests that interactions between this segment, such as hydrophobic contacts between L258 and L218 of helix H2' and among W260 and Q309 and F312 of helix H6', drive its folding.

Intrinsically disordered motifs, domains and proteins are useful in macromolecular interactions when speed, a relaxed specificity and binding to multiple partners are needed [15]. Intrinsically disordered domains appear to be relatively common in proteins that regulate transcription [16]. For example, the transcription of the toxin-antitoxin pair DOC/PHD in bacteria is regulated by the allosteric binding of an intrinsically unfolded domain [17] and moreover, the interferon-regulated transcription factor IRF-1, which is also a tumor suppressor, contains a disordered domain that serves as a binding interface for several regulatory proteins [18].

The intrinsic disorder of the tTFIIB internal loop and C-terminal extension reported here supports our proposal that they could serve as binding sites for additional factors that aid transcription initiation in *Trypanosoma brucei* [4]. These segments could be carrying out similar functions in *T. cruzi* since they are predicted by sequence analysis to also be intrinsically disordered. *T. cruzi* is the causative agent of Chagas disease, a debilitating and sometimes fatal illness that affects 16 - 18 million Latin Americans, for which no vaccine and no completely effective treatment are available. As these polypeptide segments are absent in human TFIIB, they can be considered to be useful targets for developing more selective and effective therapeutics.

Acknowledgements

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Table 1- NMR Chemical Shifts of the cTFIIB loop domain peptide

Residue	^{1}HN	^{1}H-^{13}Cα	^{1}H-^{13}Cβ	others
Gln 1		4.14	2.15, 2.13	2.39 Hγ, 6.95 Hε12, 7.64 Hε22
		54.9	**29.3**	**32.7 ^{13}Cγ**
Thr 2	8.87	4.36	4.13	1.19 Hγ
		62.2	**69.8**	**21.2**
Leu 3	8.66	4.37	1.56,1.37	1.60 γ, 0.91 δ1, 0.84 δ2
		55.0	**42.2**	**27.1, 25.0, 23.3**
Ser 4	8.42	4.38	3.87, 3.82	
		58.4	**63.8**	
Trp 5	8.20	4.65	3.29	10.18 HNε1, 7.24 Hδ1, 7.45 Hζ2,
		57.6	**29.4**	7.13 Hη2, 7.22 Hζ3, 7.57 Hε3
Glu 6	8.29	4.11	1.93, 1.83	2.08, 2.05 Hγ
		57.0	**30.0**	**35.9**
Gln 7	8.29	4.18	2.11, 2.00	2.36 Hγ, 6.93 Hε12, 7.60 Hε22
		56.3	**29.3**	**33.8**
Gly 8	8.45	3.98		
		45.3		
Thr 9	8.04	4.29	4.14	1.14 γ
		62.0	**69.9**	**21.5**
His 10	8.61	4.68	3.20, 3.09	7.14 Hδ1, 8.43 Hε2
		55.2	**28.8**	
Ile 11	8.28	4.11	1.82	1.43, 1.14 γ2, 0.89 γ3, 0.83 δ
		61.0	**38.8**	**27.2, 17.4, 12.7**
Ser 12	8.59	4.48	3.85	
		58.1	**63.8**	
Lys 13	8.58	4.35	1.88, 1.77	1.45 γ, 1.68 δ, 2.98 ε
		56.4	**33.1**	**24.8 29.0 41.8**
Gly 14	8.56	3.98		
		45.3		
Lys 15	8.39	4.35	1.87, 1.76	1.43 γ, 1.68 δ, 2.98 ε
		56.4	**33.1**	**24.7, 29.0, 41.8**
Glu 16	8.77	4.27	2.10, 1.98	2.32 γ
		57.1	**29.6**	**36.1**
Ser 17	8.28	4.44	3.91, 3.84	
		58.3	**63.9**	
Asp 18	8.39	4.67	2.77, 2.64	
		54.4	**40.9**	
Leu 19	7.83	4.17	1.59	1.56 γ, 0.91 δ1, 0.87 δ2
		56.7	**43.3**	**37.2, 25.2, 23.4**

^{1}H chemical shifts are shown in plain text and ^{13}C chemical shifts are in bold text.

Table 2- NMR Chemical Shifts of the cTFIIB C-terminal domain

Residue	^{1}H-^{15}N	^{1}H-$^{13}C\alpha$	^{1}H-$^{13}C\beta$	others
Asp 1		4.32	2.89, 2.81	
		53.4	**39.9**	
Ser 2	8.93	4.50	3.93	
	116.7	**58.6**	**63.6**	
Gly 3	8.60	3.98		
	111.2	**45.0**		
Glu 4	8.29	4.61	2.05, 1.92	2.30 γ
	121.9	**54.4**	**29.6**	**36.0**
Pro 5		4.51	2.32	2.05, 1.95 γ 3.80, 3.79 δ
		57.6	**32.1**	**27.5, 50.7**
Thr 6	8.35	4.28	4.24	1.25 γ
	114.6	**62.0**	**69.7**	**21.6**
Ala 7	8.43	4.32	1.42	
	126.4	**52.8**	**19.2**	
Gly 8	8.46	3.94		
	108.6	**45.1**		
Lys 9	8.19	4.33	1.84, 1.75	1.44γ, 1.69δ, 3.00ε, 7.60ζ
	121.1	**56.2**	**33.0**	**24.8, 29.0, 41.8**
Arg 10	8.46	4.32	1.83, 1.78	1.64γ, 3.21δ, 7.27ε
	123.2	**56.1**	**30.9**	**27.2, 43.2**
Lys 11	8.57	4.30	1.84, 1.75	1.44γ, 1.69δ, 3.00ε, 7.60ζ
	124.4	**56.2**	**33.0**	**24.8, 29.0, 41.8**
Val 12	8.35	4.10	2.05	0.92 γ
	122.9	**62.2**	**32.9**	**21.2**
Asp 13	8.54	4.63	2.74, 2.62	
	125.3	**54.0**	**41.44**	
Lys 14	8.51	4.27	1.84, 1.78	1.44γ, 1.69δ, 3.00ε, 7.60ζ
	122.6	**56.7**	**33.0**	**24.8, 29.0, 41.8**
Asn 15	8.62	4.73	2.86, 2.79	7.03 δ, H12, 7.78 δ, H22
	119.6	**53.5**	**38.8**	*113.8 Nδ*
Ser 16	8.25	4.45	3.88	
	116.2	**58.3**	**63.7**	
Glu 17	8.39	4.61	2.06, 1.92	2.31 γ
	123.9	**54.5**	**30.1**	**36.0**
Pro 18		4.42	2.30	2.06 γ, 3.82, 3.71 δ
		63.3	**32.1**	**27.5, 50.7**
Glu 19	8.66	4.23	2.05, 1.96	2.13 γ
	121.5	**56.6**	**30.1**	**36.0**
Ala 20	8.50	4.34	1.42	
	126.6	**52.6**	**19.1**	
Ser 21	8.47	**4.44**	**3.93**	
	115.6	**53.7**	**63.6**	
Gly 22	8.56	4.03		
	111.2	**45.4**		
Ser 23	8.30	4.50	3.93, 3.88	
	115.7	**58.4**	**63.7**	
Thr 24	8.28	4.34	4.25	1.22
	116.5	**62.12**	**69.5**	**21.6**
Lys 25	8.37	**4.31**	1.84, 1.78	1.44γ, 1.69δ, 3.00ε, 7.60ζ
	124.3	**56.2**	**33.0**	**24.8, 29.0, 41.8**
Arg 26	8.45	4.36	1.82, 1.76	1.64γ, 3.21δ, 7.27ε
	123.6	**55.9**	**30.9**	**27.2, 43.2**
Val 27	8.34	4.12	2.05	0.95 γ1, 0.92 γ2
	122.6	**62.0**	**32.9**	**20.6, 20.4**
Lys 28	8.53	4.30	1.84, 1.78	1.44γ, 1.69δ, 3.00ε, 7.60ζ
	122.9	**56.3**	**33.0**	**24.8, 29.0, 41.8**
Arg 29	8.59	4.31	1.81, 1.76	1.67 γ, 1.65γ, 3.21δ, 7.27ε
	124.5	**56.1**	**30.9**	**27.2, 43.2**
Glu 30	8.69	4.32	2.08, 1.94	2.29 γ
	123.0	**56.4**	**30.2**	**36.2**
Glu 31	8.59	4.32	2.14, 1.94	2.34, 2.29 γ
	122.8	**56.4**	**30.3**	**36.2**
Thr 32	7.93	4.15	4.24	1.18 γ
	120.5	**63.3**	**70.7**	**22.0**

*^{1}H chemical shifts are shown in plain text, **^{13}C** chemical shifts are in bold text, and ^{15}N chemical shifts are shown in italics.*

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