Disorder and Partial Folding in the Regulatory Subunit Hinge Region of
Trypanosoma brucei Protein Kinase A:
The C-linker Portion Inhibits the Parasite’s Protein Kinase A

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Abbreviations

cAMP, cyclic Adenosine Monophosphate; CD, Circular Dichroism; CNB, Cyclic Nucleotide Binding; COSY, Correlated Spectroscopy; DPC, Dodecylphosphocholine; DSS, 2,2-dimethyl-2-silapentane sulfonate; HSQC, Heteronuclear Single Quantum Coherence spectroscopy; IDRs, Intrinsically Disordered Regions; IS, Inhibition Sequence; NMR, Nuclear Magnetic Nuclear; NOE, Nuclear Overhauser Effect; NOESY, Nuclear Overhauser Effect Spectroscopy; PKA, Protein Kinase A; PKAc, Catalytic Subunit of the PKA; PKAr, Regulatory Subunit of the PKA; PKI, Protein kinase A inhibitor peptide; TbPKAr, Regulatory subunit of the PKA from Trypanosoma brucei; TeqPKAc, Catalytic subunit of the PKA from Trypanosoma equiperdum; TOCSY, Total Correlated Spectroscopy.

Database

Protein Kinase A: EC 2.7.11.11

Keywords

Intrinsically Disordered Regions, Regulatory Subunit of the PKA, Hinge region, CD and NMR spectroscopy, Trypanosoma brucei.
Conflicts of interest
The authors declare that there is no conflict of interests regarding the publication of this article.

Abstract
Microbial pathogens, such as Trypanosoma brucei, have an enormous impact on global health and economic systems. Protein kinase A of T. brucei is an attractive drug target as it is an essential enzyme which differs significantly from its human homolog. The hinge region of this protein’s regulatory domain is vital for enzymatic function, but its conformation is unknown. Here, the secondary structure of this region has been characterized using NMR and CD spectrosocopies. More specifically, three overlapping peptides corresponding to residues T187-I211, G198-Y223 and V220-S245 called peptide 1, peptide 2 and peptide 3, respectively, were studied. The peptide 1 and peptide 2 are chiefly unfolded; only low populations (<10%) of α-helix were detected under the conditions studied. In contrast, the peptide 3 contains a long α-helix whose population is significantly higher; namely, 36% under the conditions studied. Utilizing the dihedral φ and ψ angles calculated on the basis of the NMR data, the conformation of the peptide 3 was calculated and revealed an α-helix spanning residues E230-N241. This α-helix showed amphiphilicity and reversible unfolding and refolding upon heating and cooling. Most fascinating, however, is its capacity to inhibit the activity of the catalytic domain of Trypanosoma equiperdum protein kinase A even though it is quite distinct from the canonical inhibitor motif. Based on this property, we advance that peptoids based on the peptide 3 α-helix could be novel leads for developing anti-trypanosomal therapeutics.

1. Introduction
Kinetoplastid parasites cause different diseases that severely affect the human health. Sleeping sickness (caused by pathogenic subspecies of Trypanosoma brucei), Chagas disease
(caused by *Trypanosoma cruzi*) and the Leishmaniases (caused by *Leishmania spp*) are the major human diseases caused by kinetoplastids [1]. According to the World Health Organization "sleeping sickness", a disease of tropical Africa that is transmitted by the tsetse fly, affects more than 60 million persons in 36 countries of sub-Saharan Africa. In many of these countries, sleeping sickness is currently epidemic, and in some it is a greater cause of morbidity than HIV/AIDS [1]. Effective treatments are available but must be administered by injection and have serious side effects including death in a small percentage of cases (World Health Organization 2017).

One promising approach to develop novel inhibitors for trypanosomal proteins which are essential to the parasite but distinct from human proteins. Such inhibitors should have fewer side effects. For example, the C-terminal domain of TFIIB, an essential protein, has a surface charge distribution that is very different with respect to the human homolog [2].

Another promising target is Protein Kinase A. Existing in eukaryotic cells as a heterotetrameric holoenzyme consisting of two catalytic (C) and two regulatory (R) subunits, Protein Kinase A (PKA) regulates fundamental pathways in many organisms. Catalytic PKA subunits nearly exclusively consist of a kinase domain and their function is restricted to their kinase activity. Regulatory PKA subunits direct PKA activity by binding and inhibiting the catalytic subunits in the absence of cAMP. This binding is mainly mediated by a conserved inhibitor sequence (IS) stretch in the R subunit (R-R-X-[A/T/S]-Φ; Φ=hydrophobic residue) that also exists in PKA substrates and pseudosubstrate sequences. The linkers flanking the inhibitor sequence in the PKA regulatory subunit and joining the very stable dimerization domain (D/D) to the well-folded cyclic nucleotide-binding domains (CNB) are classified as intrinsically disordered regions (IDRs) [3]. The genomes of *Trypanosoma brucei, Trypanosoma cruzi, Leishmania major* and *Trypanosoma evansi*, contain a gene homologous to that encoding the mammalian PKA regulatory subunits. It has been suggested that this PKA-like regulatory subunit might be involved in crucial cellular processes in the trypanosomatids. In fact, the blockade of PKA function by a PKA specific inhibitor, such as H89, is lethal for *Trypanosoma cruzi*, indicating that PKA activity is critical for the viability of this hemoparasite [4]. A study of the PKA-like regulatory subunit from *Trypanosoma brucei* using homology modeling found a leucine-rich repeat domain in its N-terminal region
and revealed that the hinge region contains a sequence motif (R-R-T-T-V) that resembles the PKA IS pseudo-substrate motif: R-R-X-[A/T/S]-Φ [5] (Fig. 1). This hinge region was predicted to be rather disordered [6] and should bind to the active site of the catalytic subunit, inactivating it. The C-terminus of the regulatory subunit contains two adjacent putative Cyclic Nucleotide Binding domains (CNB) [5] (Fig. 1). The hinge region formed by two linker segments (N-linker and C-linker) and separated by an inhibition sequence may be a potential therapeutic target in *Trypanosoma brucei* because this sequence is quite distinct from its human counterpart (Figs. 1 and 2).

In this study, we structurally characterized three overlapping peptides corresponding to the hinge region of the PKA-like regulatory subunit protein from *Trypanosoma brucei* using Circular Dichroism (CD) and Nuclear Magnetic Resonance (NMR) spectroscopies. Whereas the N-terminal portion of the hinge region is intrinsically disordered, the C-terminal portion adopts a well populated α-helix in aqueous solution as well as in the presence of dodecylphosphocholine (DPC) micelles. DPC is an excellent surfactant and its micelles mimic some aspects of membrane environments [7]. We also discovered that the C-terminal portion of the hinge region specifically inhibited the PKA catalytic subunit-like of *Trypanosoma equiperdum* even though it lacks the canonical IS. This suggests that it could serve for developing inhibitors specific for trypanosomal PKA as an avenue towards novel therapeutics.

**2. Materials and methods**

**2.1. Synthetic peptides**

Three synthetic peptides were acquired from Peptide Synthesis Core Facility of the Pompeu Fabra University. The peptide number one had the sequence: TQTSADLTAIGGGRKRRTTVRGEGI of 25 residues from N-terminus of linker region. The peptide number two had the sequence: GGRKRRRTTVRGEGIDPEKAKSYVAPY was composed of 26 residues from middle of linker region. The inhibition sequence (IS) is present in both peptides 1 and 2 and is shown in bold. The peptide number three contained 26 residues and had the sequence: VAPYFEKSEDETALILKLLTYNVLFS from C-terminus of linker region. These
peptides are in hinge region of the PKA regulatory subunit-like from Trypanosoma brucei, and correspond to residues 187-245 in the full length protein. Additional peptide 3 was purchased from Genescrypt Inc. All peptides were amidated at the C-terminus, were over 95 % pure as judged by HPLC and their identities were confirmed by MALDI-TOF mass spectrometry. Deuterated dodecylphosphorylcholine-d38 (98 % atom D) (DPC) was obtained from Cambridge Isotope Labs.

2.2. Sequence analysis

PKAr from Homo sapiens RIalpha (NCBI: P10644), Rlbeta (NCBI: P31321), RIIalpha (NCBI: P13861) and RIIbeta (NCBI: P31323) were collected from National Center for Biotechnology Information GenBank. PKA regulatory subunit from Trypanosoma brucei (Tritrypdb: Tb11.02.2210), Trypanosoma evansi (Tritrypdb: TevSTIB805.11_01.4760), Trypanosoma brucei gambiense (Tritrypdb: Tbg972.11.5260, Trypanosoma congolense (Tritrypdb: TcIL3000.11.4690), Trypanosoma cruzi (Tritrypdb: TcCLB.506227.150), Trypanosoma grayi (Tritrypdb: Tgr.1276.1010), Trypanosoma vivax (Tritrypdb: TvY486_1104700) were collected from functional genomic database for pathogens of the family Trypanosomatidae TriTrypDB. The hinge region of each sequence was obtained from the difference between the full-length sequence and the predicted domains by SMART (smart.embl-heidelberg.de). Multiple sequence analysis of the hinge region was performed by Clustal Omega using the default parameters. Finally, the structure of the hinge region was predicted with the program RaptorX [8].

2.3. CD spectroscopy

A Jasco 810 spectropolarimeter 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. Three scans were acquired and averaged per spectra. The peptides were dissolved in deionized water to a concentration of 0.1 mM. Spectra were obtained at pH 5.0 and 35.0 °C. The percentage of α-helical conformation in the peptides was estimated using the CCA+ program (http://www.chem.elte.hu/departments/protnmr/cca/).

2.4. NMR Spectroscopy
All NMR spectra were performed in a Bruker AV-600 NMR spectrometer equipped with a cryoprobe. Samples contained peptides at 1.8 to 1.9 mM concentration, dissolved in H$_2$O/D$_2$O: 90% / 10% and 0.04 mM sodium 2,2 dimethyl-2-silapentane sulfonate (DSS) as the internal $^1$H chemical shift reference. The DSS signal was also used to indirectly reference the $^{13}$C chemical shift. Some samples also contained with 30 mM deuterated DPC. COSY [9], TOCSY [10,11], NOESY [12], and $^1$H-$^1$C-HSQC [13] were acquired with standard pulse sequences. The HSQC spectra were recorded using the natural abundance of $^{13}$C. To test the effect of ionic strength on the helical content of peptide 3, additional $^1$H-$^1$C HSQC spectra were recorded in 100% D$_2$O and 30 mM DPC at pH* 7.57 (where pH* is the pH meter reading without correction for the deuterium isotope effect) at 0 mM, 75 mM and 150 mM NaCl. Water suppression was accomplished by presaturation or by using the WATERGATE module [14]. Mixing times were of 60 ms in TOCSY and 150 ms in NOESY experiments. All data were acquired and processed with TOPSPIN (version 2.1) (Bruker, Germany) at different temperatures, 5 °C, 25 °C and 35 °C for the peptides. The assignment of the peptide spectra was determined by following the well established sequential specific methods on the bases of homonuclear and heteronuclear spectra [15]. The resonance signals were assigned with the aid of the program SPARKY [16].

For peptide 3 samples in 100 % D$_2$O containing 30 mM DPC, NMR diffusion ordered spectroscopy (DOSY) [17] was applied to assess the sample components’ diffusion coefficients. One sample was at pH* 4.95 and contained 150 mM NaCl. The second sample contained no NaCl and had a pH* of 7.57. The Bruker pulse program ledbpgp1d was used to calibrate d20 (150 ms) and p30 (2.1 ms). Then, a pseudo 2D experiment consisting of 32 1D spectra, recorded with 32 scans each, were recording using a pulsed field gradient whose intensity ranged from 5% to 98%. The spectra were processed with Bruker Topspin 4.0.8 and analyzed utilizing Bruker Dynamics Center 2.5.

2.5. Assignment of Proline $^1$H$\beta$ versus $^1$H$\gamma$ and Lysine $^1$H$\beta$ versus $^1$H$\delta$

The $^1$H$\beta$ and $^1$H$\gamma$ of proline residues and the $^1$H$\beta$ and $^1$H$\delta$ of lysine residues are difficult to distinguish and assign by 2D TOCSY and NOESY NMR spectra because their chemical shift values are very similar. Therefore, to unambiguously assign these nuclei, COSY spectra,
which only show crosspeaks for $^1$H on neighboring carbons rather than all the $^1$H belonging to the complete Pro or Lys side chain, were recorded and analyzed (Sup. Fig. 1).

2.6. Estimation of helix populations

The helix populations in the peptides were quantified from the chemical shift values ($\delta$) the $^1$H$_a$, $^{13}$C$_a$ and $^{13}$C$_\beta$ by comparison with the values reported in random-coil peptides [18]. To obtain the helix percentage the conformational shifts of protons ($\Delta \delta_{H_a}$) and carbons ($\Delta \delta_{C_a}$) are calculated, $\Delta \delta_{H_a} = \delta_{H_a}(\text{observed}) - \delta_{H_a}(\text{random coil})$, ppm and $\Delta \delta_{C_a} = \delta_{C_a}(\text{observed}) - \delta_{C_a}(\text{random coil})$, ppm. Values averaged for all the helical residues were divided by the $\Delta \delta_{H_a}$ and $\Delta \delta_{C_a}$ values corresponding to 100% helix, -0.39 ppm [19] and +3.09 ppm [20] respectively, and then multiplied by 100. Whereas excellent methods such as PECAN [21] have been developed to assess secondary structure in folded proteins based on chemical shifts, since the application of such programs to peptides is less clear, we have preferred to use the conformational shift approach described above.

2.7. Structure calculations

The structures of peptides 1 and 2 were not calculated because their content of ordered structure is very low. However, the structure of peptide 3 in DPC micelles was calculated. Distance constraints were derived from NOESY. The NOE cross peaks were integrated by using the automatic subroutine of the SPARKY [16], and then calibrated and converted to upper limit distance constrains with CALIBA program [22]. $\phi$ and $\psi$ angle restraints were derived from $^1$H$_a$, $^{13}$C$_a$, and $^{13}$C$_\beta$ chemical shifts (when available) by using the TALOS program [23]. Structures were calculated using the CYANA program [24]. Interactive cycles of NOE assignment and calculation families of 25 structures were performed until good agreement with the experimental constraints was achieved. All structures were visualized and analyzed with MOLMOL [25].

2.8. PKA activity using an electrophoretic gel-shift assay

PKA activity was measured using the commercial Pep-Tag® non-radioactive kit (Promega) containing the fluorescently labeled kemptide (PepTag® A1 Peptide-LRRASLG) as substrate. Kemptide is phosphorylated by PKA and the addition of negative charge alters its
motion upon electrophoresis. Assays were performed following the instructions of the company, and the reaction mixtures were loaded on a 1.2% agarose gel and separated by electrophoresis. The inhibitor PKI (5-22), was employed as a positive control. To obtain qualitative results, gels were scanned with UV light using a phototransilluminator (Fotodyne).

2.9. Partial purification of the PKA-like from Trypanosoma equiperdum

Freshly purified parasites (~10⁹–10¹⁰ trypanosomes) obtained according to Guevara et al., 2018 [26] and Lanham and Godfrey, 1970 [27], were incubated in the absence of glucose for 1 h. Cells were extracted with lysis buffer, composed of: 50 mM Tris-HCl (pH 8.0), 1 mM CaCl₂, 1 mM MgCl₂, 50 μM phenyl methyl sulfonyl fluoride (PMSF), 1 mM benzamidine, and 10 μM L-trans-epoxysuccinylleucylamido(4-guanidino)butane (E-64), by sonication, using four cycles of 30 s each, with resting intervals of 2 min per cycle. The homogenate was centrifuged at 100 000 g for 1 h, at 4 °C, in order to obtain the corresponding parasite soluble fraction, which was loaded on a 60 ml DEAE-Sepharose column that had been previously equilibrated with 50 mM Tris-HCl (pH 7.0), 0.1 mM EDTA and 0.1 mM EGTA. Proteins were eluted from the column using a 0.01–1.0 M NaCl gradient in the same buffer containing 50 μM PMSF, 1 mM benzamidine and 10 μM E-64 and a sample of each fraction was assayed using the kemptide Pep-Tag® non-radioactive kit and by Western blot using the PKA α cat antibody sc-28315 (Santa Cruz Biotechnology) (Sup. Fig. 2). Fractions containing the PKA activity peak and the protein as detected by Western blot were pooled and concentrated. To lower the NaCl concentration, the concentrated sample was then applied to a 20 mL gel filtration column (Sephacryl S-300) which had been pre-equilibrated in 50 mM Tris-HCl (pH 7.5), 5 mM NaCl, 3 mM magnesium acetate, 2 mM CaCl₂, 5 mM β-mercaptoethanol, 50 μM PMSF, 1 mM benzamidine and 10 μM E-64. The fractions obtained were stored at –80 °C in this final buffer until further use.

3. Results
3.1. Sequence analysis reveals the conservation of the pseudo-substrate inhibition motif

The sequence of the hinge region from the regulatory-like subunit of PKA from *Trypanosoma brucei* (TbPKAr-like) was aligned against homologous PKAr sequences containing this same subunit from *Trypanosoma evansi*, *T. brucei gambiense*, *T. congolense*, *T. cruzi*, *T. grayi* and *T. vivax* as well as human PKAr. Whereas the hinge sequences of *T. brucei*, *T. evansi* and *T. brucei gambiense* are 100% identical, in *T. congolense*, *T. cruzi*, *T. grayi* and *T. vivax*, the levels of sequence identity are significantly lower (Fig. 2). Remarkably, the RRX(T/S)V segment is conserved in all Trypanosomal sequences. This segment, which functions as a pseudo-substrate inhibition motif in all PKAr isoforms, is somewhat distinct from the human RIIα and RIIβ subdomain IS (RRX(A)y), where y is any hydrophobic residue. In particular, note that Thr or Ser, which can be phosphorylated in the Trypanosomal proteins and human RIIα and RIIβ, are replaced by Ala or Gly, whose side chain cannot be post-translationally modified, in these two RI human isoforms (Fig. 2). The structural analysis of the hinge region performed using the program RaptorX predicts that the hinge region would be disordered except for an α-helix composed of residues S227-L243 (Table S1).

3.2. Circular dichroism spectroscopy detects helix in peptide 3

The backbone conformation of the three peptides in water without surfactant was probed using far-UV circular dichroism spectroscopy. The CD spectra of peptides 1 and 2 show minima between 195 and 200 nm (Fig. 3), which is characteristic of statistical coils [28, 29, 30]. In contrast, peptide 3, which corresponds to the C-terminal zone of the hinge, shows a maximum at 195 nm, a deep minimum at 210 nm and a more shallow minimum near 222 nm. These are spectral features of a mixture of helical conformations with statistical coil. The quantitative analysis of these data confirms that whereas peptides 1 and 2 are mostly disordered, peptide 3 is about 36% helical under these experimental conditions (Table 1). The presence of this structure will be corroborated in the next section by NMR spectroscopy.

The thermal denaturation of the peptide 3 (C-linker segment) was monitored by CD spectroscopy by monitoring the signal at 220 nm as the temperature was increased from 5 ºC to 90 ºC (Fig. 4). The signal gradually increased during the heating process, which strongly suggests the absence of a well folded domain undergoing cooperative two-state denaturation. Interestingly, the observation of significant negative ellipticity at 90 ºC suggests that a
partially folded state may persist at high temperature (Fig. 4, A,B). Afterwards, upon cooling to 5 °C, the ellipticity at 220 nm progressively decreased to values near those observed prior to heating, suggesting that the thermal denaturation and renaturation of peptide 3 are reversible (Fig. 4, C,D).

3.3. Conformation of the peptide 1

1D and 2D NMR spectra were recorded on peptide 1, the N-linker segment of the hinge region of the T. brucei PKA regulatory subunit in water and in the presence of DPC micelles. The amide $^1$H region (7.6 – 9.0 ppm) of the $^1$H NMR spectra in water at pH 5.1 and 5 °C shows little dispersion (Sup. Fig. 3A), suggesting that this peptide does not adopt a stable secondary structure in these conditions. The complete $^1$HN-$^1$Hα assignments of peptide 1 in the presence of DPC micelles are shown Sup. Fig 4. The full set of assignments are listed in Sup. Table 2 and the assignments of peptide 1, 2 and 3 have been deposited in the BMRB database under entry number 50597. Interestingly, the analysis of the $^1$Hα, $^{13}$Cα and $^{13}$Cβ conformational chemical shifts indicates that residues S4 to I10 do tend to adopt a partial helical conformation (Helix I), although its population is rather low (Fig. 5A and Table 1). The population of helical structure is estimated to be 19 % for these residues (Table 1). The rest of peptide 1 does not adopt any detectable structure. Overall, the population of helical structure in peptide 1 calculated to be 5% and 8% based on the $^1$Hα and $^{13}$Cα chemical shifts, respectively (Table 1). The position of this short helix in peptide 1 coincides with structural models of the T. brucei PKA regulatory subunit based on programs which predict secondary structure [6].

The $^1$H NMR spectrum of peptide 1 in the presence of DPC micelles also shows poor dispersion in the $^1$H amide region, (Sup. Fig 5A). The analysis of the $^1$Hα, $^{13}$Cα and $^{13}$Cβ conformational chemical shifts, shown in Fig. 5A and Table 1, indicate that peptide 1 in DPC micelles has the same tendency to form a helical structure as in aqueous solution. Moreover, taking the $^1$HN signal of S4 at 8.6 ppm in water as a local reference for peptide 1, the lack of change in the chemical shifts suggests that the presence of the DPC micelles does not alter the chemical environment of peptide 1 (Sup. Fig. 6).

3.4. Conformation of the peptide 2
Next, we recorded 1D and 2D NMR spectra on peptide 2. The 1D $^1$H NMR spectrum in aqueous solution at pH 5.0, 5 ºC shows little dispersion in the amide $^1$H region (8.50 - 8.75 ppm) (Sup. Fig. 3B), suggesting that peptide 2, like peptide 1, does not adopt a stable secondary structure. The complete $^1$HN-$^1$H$_{\alpha}$ assignment of peptide 2 is shown in Sup. Fig. 7 and all the assignments are listed in Sup. Table 3. However, the analysis of the $^1$H$_{\alpha}$, $^{13}$C$_{\alpha}$ and $^{13}$C$_{\beta}$ conformational chemical shifts, shown in Fig. 5B and Table 1, indicate that the residues spanning P16 to Y22 in peptide 2 tend to adopt some helical structure (Helix II), whose population is calculated to be 30%. The position of this helix in peptide 2 concurs with the structural models reported recently for this region in the T. brucei PKA regulatory subunit [6]. The rest of the peptide does not adopt any preferred conformation. The population of helix for the whole peptide is estimated to 2% and 18% based on the $^1$H$_{\alpha}$ and $^{13}$C$_{\alpha}$ conformational chemical shifts, respectively. The 1D $^1$H NMR spectrum of peptide 2 recorded in the presence of DPC micelles also reveals poor signal dispersion in the amide region, as shown in Sup. Fig. 5B. Interestingly, the presence of DPC micelles shifts all the $^1$HN resonances to higher field, including the signals of the glycines. This is shown in Sup. Fig. 8 which uses the $^1$HN signal of S21 at 8.24 ppm as a local reference. The analysis of the $^1$H$_{\alpha}$, $^{13}$C$_{\alpha}$ and $^{13}$C$_{\beta}$ conformational chemical shifts, displayed in Fig 5B and Table 1, indicates that peptide 2 partially adopts a helical conformation. The helical population is 22% considering the whole peptide and reaches 53% for the segment spanning residues P16 to Y22 (Table 1).

3.5. Conformation of the peptide 3

The peptide 3 (C-linker) was sparingly soluble in water and did not afford NMR spectra of sufficient quality for analysis. Nevertheless, the peptide 3 dissolved completely in 30 mM DPC. DOSY spectra were recorded to determine the diffusion coefficients and thereby assess the interaction of peptide 3 and DPC. The results, which are summarized in Table S5, show a good agreement between the diffusion coefficients of DPC and peptide 3. This strongly suggests that the molecules of peptide 3 are bound to the DPC micelles. The signals of $^1$HN in the 1D $^1$H spectra recorded at pH 5.0, 25ºC showed good dispersion (Sup. Fig. 5C, Fig. 6A), which suggest the presence of secondary structure. A long span of peptide 3 corresponding to residues S227–L243 have $^1$HN conformational chemical shifts ($\Delta\delta$ $^1$H) < -
0.10 ppm, which is indicative of α-helix (Fig. 5C). To corroborate this finding, the $^{13}$Cα and $^{13}$Cβ signals in a $^1$H-$^{13}$C HSQC spectrum were analyzed and assigned for most residues (Fig. 6B and Sup. Table 4). Values of $\Delta\delta^{13}$Cα > 0.70 ppm for residues spanning S227–L243 and of $\Delta\delta^{13}$Cβ < -0.70 ppm for the segment composed of residues D228–V242 support the presence of α-helical conformers (Fig. 5C). Based on the magnitude of these conformational chemical shifts, this α-helix (helix III) would have a population of 48% to 61% in 30 mM DPC and would include most of peptide 3. These results are summarized in Table 1. Additional $^1$H-$^{13}$C HSQC spectra of peptide 3 showed little variation over NaCl concentrations varying from 0 to 150 mM, which strongly suggests that the α-helix is largely maintained at physiological ionic strength (Sup. Fig. 9).

3.6. Analysis of NOE Data

The NOE signals that were important to characterize the secondary structure of peptides 1, 2 and 3 are summarized in Fig. 7. Whereas peptides 1 and 2 gave rise to many αN (i, i+1) NOEs, only a few weak medium range (i, i+3) and no (i, i+4) NOE signals were observed (Fig. 7A,B). The weak (i, i+3) NOEs do coincide with the zone of sparsely populated helical structure as detected by conformational shift analysis. However, the low number of medium range NOEs concords with the dearth of secondary structure in peptides 1 and 2 as evinced by their conformational chemical shifts. Due to their low populations, no structural calculations were attempted for peptides 1 or 2.

By contrast, peptide 3 showed more medium range NOEs; namely 2 αN (i, i+2), 3 NN(i, i+2) and 2 αN(i, i+3) NOEs (Fig. 7C) which indicate the presence of preferred conformations between residues S8 and L24. This is in agreement with the zone of α-helical structure detected by conformational chemical shift analysis. The observation of additional NOEs between L16 $^1$Hα and T20 $^1$HN, D10 $^1$Hα and A13 $^1$Hβ and T12 $^1$Hα and L15 $^1$Hβ provides more spectroscopic evidence for an α-helix forming between residues 8 and 24 (Fig. 7C). Moreover, a large number of NN (i, i+1) signals (Fig. 8) are observed; these are characteristic of helical conformations or tight turns.

3.7. 3D Model of the peptide 3 in DPC
To visualize the conformational ensemble, a family of conformers of the peptide 3 in DPC was calculated using the program CYANA and the twenty lowest energy structures are shown in Fig. 9. Whereas residues 1-8 are disordered, the peptide 3 adopts an α-helical conformation spanning residues D9 to V23 (Helix III). The structural superposition for residues E11 to N22 in the center of the α-helix shows an RMSD of 0.61 Å for the 20 lowest energy structures. As expected, most of the ϕ, ψ dihedral angles map to the α-helical region of the Ramachandran map (Sup. Fig. 10). Interestingly, the nonpolar residues L16, L19 and V23 are localized on the same face of the α-helix. Since the polar residues K17 and Y21 are positioned on the opposite face, this α-helix has a certain amphiphilic character (Fig. 9B).

3.8. Peptide 3 selectively inhibits the PKA-like from Trypanosoma equiperdum

The ability of peptides 1, 2 to inhibit the kinase activity of the catalytic subunits of PKA (PKAc) from Sus scrofa and peptide 3 to inhibit the kinase activity of PKA-like from T. equiperdum (TeqPKAc) was measured using the PepTag® method. Control experiments showed that both PKAc and TeqPKAc phosphorylate the substrate and that PKI (5-22) successfully blocks the action of PKAc and TeqPKAc (Fig. 10A). Peptides 1 and 2 both contain a tract of residues, RRTTV, that resembles the inhibitor sequence of PKI (RRNAI), and both peptides moderately inhibit the kinase activity of PKAc from Sus scrofa (Fig. 10A). By contrast, peptide 3 (C-linker) does not have a tract that resembles the canonical inhibitor sequence of PKI and it did not block the action of PKAc from Sus scrofa (Fig. 10A). Remarkably, peptide 3 did completely block the kinase activity of TeqPKAc (Fig. 10A). These results indicate a striking difference in the inhibition of PKAc and TeqPKAc by peptide 3. They constitute an important proof-of-concept that TeqPKAc can be selectively inhibited by peptide 3 without blocking PKAc. It is possible that the α-helix III of peptide 3 plays a role in the regulatory auto-inhibition of TeqPKAc.

By means of a dot-blot assay using antibodies specific for phosphothreonine, we found that peptides 1 and 2 with sequence: RRTTV are recognized and phosphorylated on Thr by TeqPKAc, while Peptide 3 with Thr residues but lacking the sequence (RRTTV) is not phosphorylated by TeqPKA (Fig. 10B). Finally, Peptides 1, 2 and 3 were not phosphorylated on Thr by PKAc from Sus scrofa (Fig. 10B).
4. Discussion

In this study, we have characterized three overlapping peptides from the hinge region of the PKA-like enzyme of *Trypanosoma brucei*. Since this protein is both essential to the parasite yet significantly different from the homologous protein in humans, it is a good target for developing inhibitors as therapeutics.

Our recent computational analysis of the hinge region secondary structure revealed distinct predictions from the six different secondary structure algorithms used [6], suggesting the need for experimental studies. Our spectroscopic characterization show that peptides 1 and 2 of the hinge region are chiefly disordered; each contains only one short, modestly populated α-helix. It is notable that these α-helices do not include the RRTTV inhibitor sequence, which we find to be completely disordered. This inhibitor sequence would therefore be highly accessible which could facilitate its interaction with the kinase domain. This is consistent with the results of our kinase activity assays, which showed that peptides 1 and 2 partially inhibit the catalytic action of porcine protein kinase A. Due to a lack of antibodies specific for phospho-Ser and phospho-Tyr, we can not rule out that these Ser and Tyr residues outside the inhibitor sequence may become phosphorylated. Nevertheless, considering that: 1) both peptides 1 and 2 eventually become phosphorylated on Thr residues, 2) both contain the RRTTV inhibitor sequence and 3) a similar auto-inhibitor motif in mammalian PKAs is known to act as a pseudosubstrate, it is plausible that the inhibitory action of peptides 1 and 2 is due to the RRTTV segment binding to the catalytic site of the PKAc. More importantly, we can conclude that peptides 1 and 2 and the RRTTV segment would be a poor starting point for developing *Trypanosome* specific PKA-like kinase inhibitors.

In contrast to peptides 1 and 2, peptide 3 does contain a long α-helix. A high population of α-helical conformation is detected both in aqueous solution as well as in the presence of DPC micelles by CD and NMR spectroscopies, respectively. The position of the α-helix predicted by RaptorX (residues E228-Y240) is in fairly good agreement with the experimental results (an α-helix spanning residues S227-L243). The elevated α-helix
population and its stability against thermal unfolding can be attributed to several factors. First, the segment that adopts the \( \alpha \)-helix is rich in residues, like Ala, Lys and Leu, which have a high intrinsic propensity to form \( \alpha \)-helix [31]. Secondly, the presence of a putative cation-\( \pi \) interaction between K236 and Y240 [32] as well as hydrophobic interactions between nonpolar residues spaced i, i+3 or i, i+4 [33] will stabilize the \( \alpha \)-helix. Since the hydrophobic residues are positioned on one face of the \( \alpha \)-helix, they are placed to favorably interact with the nonpolar interior of DPC micelles. Thirdly, no less than three residues as the N-terminus of the \( \alpha \)-helix (S227, D229 and T231) have high “N-cap” propensities meaning their side chains can form \( \alpha \)-helix stabilizing hydrogen bonds with the backbone HN groups in the first turn of the \( \alpha \)-helix [34]. Fourthly, there are multiple negatively charged residues near the N-terminus of the \( \alpha \)-helix which would interact favorably with the helix macrodipole [35]. The majority of the residues adopting an \( \alpha \)-helix in peptide 3 also do so in the context of X-ray crystal structure of the cAMP-independent protein kinase A from \emph{T. brucei} (PDB 6FLO) [36]. Some minor difference are likely due to tertiary contacts in the context of the full length protein (Sup. Fig. 11). The ability of residues D229-V242 to autonomously adopt a helical structure suggests that it may aid the folding of the complete enzyme [37].

It is important to emphasize that based on the sequences of the hinge loop in other \emph{Trypanosoma} PKA-like regulatory subunit kinases (Fig. 2), these \( \alpha \)-helix stabilizing interactions are always present. This strongly suggests that the sequence and the \( \alpha \)-helix it adopts play a functional role that is important enough to be selected and maintained by evolution. In contrast, based on their sequences (Fig. 2), this \( \alpha \)-helix would not be present in human PKA. One key finding here is that peptide 3 can selectively inhibit the kinase activity of the Trypanosoma PKA-like enzyme in an IS-independent manner. The report of an \( \alpha \)-helix lacking an inhibitor sequence that binds the active site and inhibits the catalytic activity of the PKA-like kinase in the protist \emph{Dictyostelium discoideum} [38], lends credence to the idea that the long \( \alpha \)-helix of the hinge region of the \emph{Trypanosoma} enzyme performs a similar function. Based on this, we advance that peptoids resembling this \( \alpha \)-helix but with improved pharmacological properties could be excellent leads for developing new anti-trypanosomal therapeutics.
Acknowledgments

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Author contributions

NAA designed research. NAA and DVL wrote the paper. NAA and MB performed research and NAA analyzed data with assistance from DVL and MB.

References


The hinge region is formed by the inhibition sequence (IS) and linker segments. The sequences of the three overlapping peptides synthesized to study the hinge region are indicated. LRR: Leucine Rich Repeat; CNB: Cyclic Nucleotide Binding domains A and B; P1: peptide 1, the N-linker; P2: peptide 2, the middle linker; P3: peptide 3, the C-linker. Note that the IS is present in both P1 and P2.
**Fig. 2.** Multiple sequence alignment of the linker region of the PKA regulatory subunits

CD spectra of peptide 1 (dashed line), peptide 2 (dotted line) and peptide 3 (solid line) from the hinge region of the protein TbPKAr. All peptides were dissolved in deionized water to a final concentration of 0.1 mM at pH 5. The spectra were recorded at 35 °C.
Fig. 4. Thermal Unfolding and Refolding of peptide 3 monitored by CD spectroscopy.

CD spectra of the peptide 3 (C-linker). (A) CD spectra of peptide 3 at 35 ºC (partially helical) and at 90 ºC (mostly denatured). (B) Temperature dependence of the ellipticity at 220 nm, which is indicative of helix, during heating. (C) CD spectra of peptide 3 at 90 ºC and then after re-cooling to 35 ºC. (D) Temperature dependence of the ellipticity at 220 nm during re-cooling.
Conformational chemical shifts ($\Delta\delta$) of the alpha proton ($^{1}\text{H}_{\alpha}$) alpha carbon ($^{13}\text{C}_{\alpha}$) and beta carbon ($^{13}\text{C}_{\beta}$) in water and in DPC micelles. (A) Peptide 1. (B) Peptide 2. (C) Peptide 3. The residues with significant populations of $\alpha$-helix are boxed. The black and gray bars shown in panels A and B correspond to the data in water and 30 mM DPC, respectively.
Fig. 6. Assignment of NMR signals of peptide 3 in DPC.

(A) Superposition of 2D $^1$H-$^1$H TOCSY (gray contours) and 2D $^1$H-$^1$H NOESY (dark red) spectra. $^1$HN-$^1$Hα crosspeaks are labeled. The dashed red line represents the sequential connectivities for residues S8 to D10 and E11 to L16. (B) The $^1$Hα-$^{13}$Cα region of the $^1$H-$^{13}$C HSQC spectra.
**Fig. 7.** Summary of the sequential NOEs in aqueous solution + 30 mM DPC micelles.

(A) Peptide 1. (B) Peptide 2. (C) Peptide 3. The width of the lines reflects the NOE intensity. Strong NOEs indicative of helical structure (*i.e.* \(NN(i, i+1), \alpha\beta(i, i+3)\) and \(\alpha N(i, i+4)\)), are only observed in peptide 3.
The spectral region near the $^1\text{HN} - ^1\text{HN}$ diagonal is shown. The NOE signals are indicative of tight turns or helical structure.
Fig. 9. The 3D structure of peptide 3 in the presence of DPC micelles

Structures were calculated using the program CYANA. (A) The family of the 20 lowest energy structures superimposed over the α-helical region spanning residues 8 to 24. (B) Ribbon diagram of the helical region; note the position of the hydrophobic (blue spheres) and polar residues (green) on opposing faces of the helix. These figures were composed using the program MOLMOL. Nt = N-terminus, Ct = C-terminus.
**Fig. 10.** Kinase inhibition assays using the PepTag® method.

(A) Left panel, kinase inhibition assays of porcine PKA catalytic subunit (PKAc): **Negative control:** 1 μL Kempide (0.1 μg/μL); **PKAc:** 1 μL Kempide + 5 μL PKAc (0.2 μg/μL); **PKAc + PKI:** 1 μL Kempide + 5 μL PKAc + 0.5 μL PKAc Inhibitor (5-22) (PKI) (0.2 μg/μL); **PKAc + Peptide 1:** 1 μL Kempide + 5 μL PKAc + 10 μL Peptide 1 (1 μg/μL); **PKAc + Peptide 2:** 1 μL Kempide + 5 μL PKAc + 10 μL Peptide 2 (1 μg/μL); **PKAc + Peptide 3:** 1 μL Kempide + 5 μL PKAc + 10 μL Peptide 3 (1 μg/μL). The arrows mark the position of the phosphorylated substrate.

Right panel, kinase inhibition assays of PKA catalytic-like subunit from *Trypanosoma equiperdum* (TeqPKAc): **Negative control:** 1 μL Kempide; **TeqPKAc:** 1 μL Kempide + 10 μL TeqPKAc (~0.05 μg/μL); **TeqPKAc + PKI:** 1 μL Kempide + 10 μL TeqPKAc + 0.5 μL PKI; **TeqPKAc + Peptide 1:** 1 μL Kempide + 10 μL TeqPKAc + 10 μL peptide 1 (1 μg/μL); **TeqPKAc + Peptide 2:** 1 μL Kempide + 10 μL TeqPKAc + 10 μL peptide 2 (1 μg/μL); **TeqPKAc + Peptide 3:** 1 μL Kempide + 10 μL TeqPKAc + 10 μL peptide 3 (1 μg/μL). The arrows mark the position of the phosphorylated substrate.

(B) Dot blot assays with anti-phosphothreonine antibodies sc-5267 (Santa Cruz Biotechnology). Left panel: membrane stained with Ponceau red. Right panel: P-Thr dot blots. **a1:** Kinase buffer; **a2:** Peptide 1; **a3:** Peptide 2; **a4:** Peptide 3; **a5:** TeqPKAc; **a6:** PKAc; **b1:** Peptide 1 + TeqPKAc; **b2:** Peptide 1 + PKAc; **b3:** Peptide 2 + TeqPKAc; **b4:** Peptide 2 + PKAc; **b5:** Peptide 3 + TeqPKAc; **b6:** Peptide 3 + PKAc. The most intense blots are boxed.
Table 1. Helix populations estimated for peptides in water and DPC Micelles.

<table>
<thead>
<tr>
<th></th>
<th>Peptide 1 (N-linker)</th>
<th>Peptide 2 (Middle-linker)</th>
<th>Peptide 3 (C-linker)</th>
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<tbody>
<tr>
<td></td>
<td>H₂O</td>
<td>DPC</td>
<td>H₂O</td>
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<tr>
<td>Helix residues (%)</td>
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<td>Circular dichroism a</td>
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<tr>
<td>ΔδH₀(α), ppm b</td>
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<td>-0.05</td>
<td>-0.02</td>
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<td>Helix population (%)</td>
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<tr>
<td>ΔδCα(α), ppm c</td>
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<tr>
<td>Helix population (%)</td>
<td>8.20</td>
<td>18.0</td>
<td>8.20</td>
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*Calculated with CD spectrum analyzer CCA+ program.

b ΔδH₀(α) value averaged for the helical residues.

c Estimated from ΔδH₀(α) values.

d ΔδCα(α) value averaged for the helical residues.

* Estimated from ΔδCα(α) values.
Table 2. Structural statistics for the families of 25 structures of Peptide 3 in DPC Micelles.

<table>
<thead>
<tr>
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<tr>
<td>1-26, backbone heavy atoms</td>
<td>3.26 ± 0.87</td>
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<tr>
<td>1-26, all heavy atoms</td>
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<tr>
<td>11-22, backbone heavy atoms</td>
<td>0.61 ± 0.39</td>
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<td>11-22, all heavy atoms</td>
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# Structures with violations

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<td>Van der Waals restraints</td>
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</tr>
<tr>
<td>Total</td>
<td>6/25</td>
</tr>
</tbody>
</table>

Ramachandran plot (11-22)

| Most favoured (%)     | 68.7 |
| Allowed (%)           | 13.5 |
| Additionally allowed (%) | 9.4  |
| Disallowed (%)        | 8.3  |