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Letter to the Editor: Sequence-specific ^{1}H, ^{13}C and ^{15}N resonance assignments of the C-terminal domain of KaiA, a circadian clock protein

Ioannis Vakonakis & Andy C. LiWang*
College of Agriculture and Life Sciences, Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843-2128, U.S.A.

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Biological context

An endogenous, self-sustaining circadian clock modulates metabolic, physiological and behavioral activities of virtually all organisms with a period of ∼24 h, and has been shown to enhance organism fitness. Cyanobacteria are the only prokaryotic group where a circadian oscillator function has been demonstrated thus far (Ishiura et al., 1998). The basic timing oscillator of the cyanobacterium *Synechococcus elongatus* (PCC 7942) consists of three proteins, KaiA, KaiB and KaiC, whose expression and mutual interactions drive the circadian rhythm (Ishiura et al., 1998; Iwasaki et al., 1999). We have recently demonstrated that KaiA consists of two domains, an N-terminal response regulator receiver-like domain whose structure we have determined, and a C-terminal KaiC interacting domain (Williams et al., 2002) which is responsible for KaiA dimerization. We have also shown that KaiA enhances the autophosphorylation function of KaiC, and that the C-terminal domain alone appears to be sufficient for that function *in vitro*. KaiC phosphorylation is known to be modulated *in vivo* in a circadian manner; therefore we expect that KaiA is a primary regulator element of KaiC phosphorylation and thus circadian rhythm.

The derived *S. elongatus* KaiA C-terminal domain exhibits a non-cooperative thermal unfolding, characteristic of molten globule proteins. In contrast the equivalent domain from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 exhibits much better cooperativity of unfolding (data not shown). This 104-residue domain, henceforth called ThKaiA180C, has 62% and 82% sequence identity and similarity, respectively, to the *S. elongatus* KaiA C-terminal domain. ThKaiA180C forms a 24.5 kD homodimer, with a disulphide bond across the dimer interface, connecting the single cystine residue of each monomer. The residue responsible for this disulphide bond, C96, is conserved between *S. elongatus* and *T. elongatus*.

*To whom correspondence should be addressed. E-mail: andy-liwang@tamu.edu

Methods and results

Protein expression and purification: The gene coding for residues 180-283 of *T. elongatus* KaiA was subcloned into pET-32a+ vector and *Escherichia coli* BL21(DE3) was transformed with the resulting plasmid. Bacteria were grown at 37 °C in minimal medium containing $^{15}NH_4Cl$ as the only nitrogen source, and with either $^{13}C_6$-glucose or unlabeled glucose. Cells were induced by making the cell culture 1 mM in IPTG and harvested by centrifugation after 5 h. The cell pellet was resuspended, passed through a French press twice and cell lysates were centrifuged at 20,000 g for 30 min. The recombinant protein was purified by metal-affinity chromatography and cleaved with enterokinase, which results in the addition of three residues (AMA) to the N-terminus of ThKaiA180C. Thioredoxin was separated from the mixture by a second metal-affinity chromatography step and ThKaiA180C was further purified by anion exchange chromatography. Protein purity was analyzed using SDS-polyacrylamide gel electrophoresis. For double labeling typically 14 mg ThKaiA180C were obtained from 1 L of culture. ThKaiA180C was readily oxidized under the NMR conditions used here in approximately 24 h, therefore we proceeded to obtain complete assignments for the oxidized form.

NMR spectroscopy: NMR samples contained 20 mM NaCl, 20 mM sodium phosphate pH 7.0 at 50 °C (pH 7.07 at 23 °C), 0.02% NaN_3, 0.1 mM DSS, 1.2 mM oxidized ThKaiA180C in a 95% H_2O/5% D_2O solvent mixture or 100% D_2O. The spectra were recorded on Varian Inova 600 MHz and 500 MHz spectrometers at 50 °C at the Biomolecular NMR Laboratory at Texas A&M University. ^{1}H, ^{13}C and

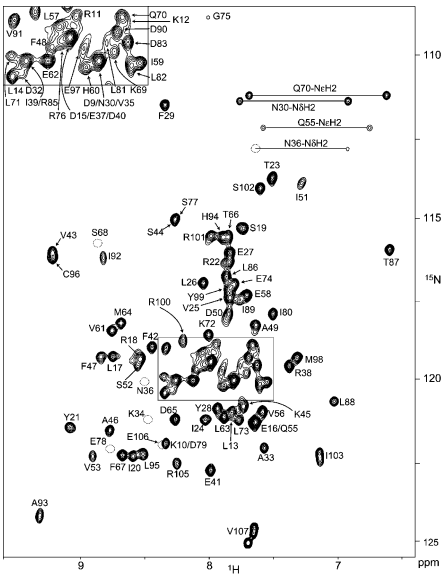

Figure 1. 2D ^{1}H, ^{15}N HSQC spectrum of ^{15}N-labeled ThKaiA180C recorded on a 600 MHz Varian Inova spectrometer at 50 °C. Amide resonances below the current contour level are indicated by dashed circles. The side chain NH_2 resonances of glutamine and asparagine residues are connected by horizontal bars. Expansion of the region enclosed by the box is shown at the upper left corner of the spectrum.

^{15}N chemical shifts were referenced to internal DSS (Markley et al., 1998).

Sequence specific backbone assignments of $^{1}H^{\alpha}$ $^{1}H^{\beta}$, $^{1}H^{N}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and ^{15}N were obtained from CBCA(CO)NH, CBCANH and HBHA(CO)NH (Grzesiek and Bax, 1993) experiments. Aliphatic assignments for the side chains were obtained from C(CO)NH, H(CCO)NH (Grzesiek et al., 1993) and H(C)CH-COSY experiments while glutamine and asparagines side chain amide assignments were taken from CBCA(CO)NH and ^{1}H, ^{15}N HSQC experiments. Additional aliphatic assignments as well as assignments of aromatic side chains were performed by combining information from H(C)CH-COSY, 4D ^{13}C–^{13}C edited NOESY and ^{1}H, ^{13}C HSQC experiments. Data processing and analysis were carried out using the software packages NMRPipe (Delaglio et al., 1995), PIPP and STAPP (Garrett et al., 1991).

Extent of assignments and data deposition

Based on the information from the heteronuclear experiments we were able to assign the entire backbone $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{1}H^{\alpha}$ and $^{1}H^{\beta}$ resonances. All $^{1}H^{N}$ and ^{15}N resonances visible in the ^{1}H, ^{15}N HSQC have also been assigned. Amide proton resonances for residues A1-A8, T31, S54 and Y84 were not observed, possibly due to fast amide proton exchange at these sites. A labeled ^{1}H, ^{15}N HSQC spectrum is shown in Figure 1. All aliphatic ^{1}H and ^{13}C resonance assignments except R85 $^{1}H^{\delta 2/\delta 3}$, all glutamine and asparagines amide side chain assignments and nearly 70% of aromatic assignments have been completed. The ^{1}H, ^{15}N and ^{13}C chemical shifts for ThKaiA180C have been deposited in the BioMagResBank under accession number 5824. Chemical shift index (Wishart and Sykes, 1994) analysis for $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{1}H^{\alpha}$ suggests the presence of four α-helices spanning residues 7–26, 35–48, 53–73 and 84–102.

We have also been able to obtain approximately 90% complete backbone $^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{1}H^{N}$ and ^{15}N resonance assignments for the reduced form of the protein, which have been deposited under BMRB accession number 5825. Since the maximum $^{13}C^{\alpha}$ chemical shift difference of the two forms is, apart from C96, only 0.29 ppm, we do not expect significant secondary structure changes as result of the oxidation.

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