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¹H, ¹³C, and ¹⁵N backbone and side chain resonance assignments of thermophilic *Geobacillus kaustophilus* cyclophilin-A

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Abstract

Cyclophilins catalyze the reversible peptidyl-prolyl isomerization of their substrates and are present across all kingdoms of life from humans to bacteria. Although numerous biological roles have now been discovered for cyclophilins, their function was initially ascribed to their chaperone-like activity in protein folding where they catalyze the often rate-limiting step of proline isomerization. This chaperone-like activity may be especially important under extreme conditions where cyclophilins are often over expressed, such as in tumors for human cyclophilins (Lee 2010), but also in organisms that thrive under extreme conditions, such as thermophilic bacteria. Moreover, the reversible nature of the peptidyl-prolyl isomerization reaction catalyzed by cyclophilins has allowed these enzymes to serve as model systems for probing the role of conformational changes during catalytic turnover (Eisenmesser, Bosco et al. 2002; Eisenmesser, Millet et al. 2005). Thus, we present here the resonance assignments of a thermophilic cyclophilin from *Geobacillus kaustophilus* derived from deep-sea sediment (Takami, Nishi et al. 2004). This thermophilic cyclophilin may now be studied at a variety of temperatures to provide insight into the comparative structure, dynamics, and catalytic mechanism of cyclophilins.

Biological context

Over the last 30 years, numerous biological roles have emerged for the cyclophilin class of enzymes including intracellular functions such as protein folding, protein trafficking, and signal transduction and extracellular roles that mimic cytokine-like functions. The chaperone-like activity of cyclophilins is particularly important in mediating protein folding

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under extreme conditions, such as in the tumor microenvironment where human cyclophilins are often overexpressed (Lee 2010) or in pathogens that have to endure harsh environments (Thai, Renesto et al. 2008). The prototypical human cyclophilin, cyclophilin-A (hCypA), has served as a model for understanding the relationship between structure, enzyme motions (i.e. dynamics), and function. The reversible nature of the peptidyl-prolyl isomerization reaction that is catalyzed by cyclophilins allows this relationship to be readily studied during turnover. Specifically, nuclear magnetic resonance (NMR) solution studies together with computational studies of hCypA have provided profound insight into our understanding of dynamic evolution (Eisenmesser, Millet et al. 2005; Doshi, McGowan et al. 2012). For example, inherent motions within hCypA have been identified within multiple regions surrounding its active site on the same micro-millisecond timescale as those that occur during catalysis of model substrates, suggesting that enzyme motions, in addition to their structural features, have been evolutionarily poised for function (Eisenmesser, Millet et al. 2005; Schlegel, Armstrong et al. 2009). However, as opposed to the initial reports that presented a highly simplified hCypA conformational exchange process on the micro-millisecond timescale (Eisenmesser, Millet et al. 2005), more recent studies utilizing multiple field strengths, deuteration, mutagenesis, and temperature variations have revealed a far more complicated array of partially coupled motions (Schlegel, Armstrong et al. 2009). Thus, there still remains an unfortunate lack of clarity regarding the mechanism of cyclophilin-mediated isomerization of its substrates and it also remains unclear as to exactly how the multiple dynamic processes identified in the human form underlie catalysis (Schlegel, Armstrong et al. 2009).

Following on such studies, a new model system that allows for dynamics and functional studies at a much wider range of temperatures than hCypA, which has limited long term stability above ~25°C, could help further our understanding of the relationships between structure, stability, dynamics, and function. For example, atomic resolution studies collected at multiple temperatures that monitor dynamics on both the enzyme and substrate would allow for the dissection of regions linked to catalysis. To this end, we have expressed, purified, and for the first time assigned the resonances of a thermophilic cyclophilin from *Geobacillus kaustophilus* (gCypA), which shares 49% similarity and 37% identity to hCypA. Unraveling the relationship between structure, dynamics, and function is particularly important for cyclophilins, since human isoforms are involved in viral infection, cancer, and inflammatory disorders (Obchoei, Wongkhan et al. 2009) and both viral and bacterial cyclophilins could potentially serve as therapeutic targets to block infectious pathogens (Obi, Nordfelth et al. 2011). Initial studies shown here indicate that while the structure of gCypA is similar to hCypA, there are shifts in some of the structural elements relative to the sequence alignment. Moreover, gCypA is highly stable at high temperatures as expected.

Method and experiments

Protein expression and purification

DNA encoding the 146 amino acid gCypA was commercially purchased (Genewiz, South Plainfield, NJ) and subsequently cloned into the pET15b plasmid encoding an N-terminal

6xHis-tag for expression in *E. Coli* strain BL21 (DE3). Cell pellets were lysed via sonication in nickel equilibration buffer (50 mM Na₂HPO₄, pH 7.5, 500 mM NaCl, 10 mM imidazole), applied to a 10–20 ml Ni-sepharose column, and eluted with nickel elution buffer (50 mM Na₂HPO₄, pH 7.5, 500 mM NaCl, 400 mM imidazole). Virtually all of the expressed gCypA was found to be soluble upon sonication. Elutions comprising 6xHis-tagged gCypA were then concentrated to approximately 2–3 ml, the thrombin tag was cleaved off with 3 units of thrombin incubated overnight at room temperature, and the protein was applied to a superose-75 sizing column equilibrated in NMR buffer (50 mM Na₂HPO₄, pH 6.5). Elutions comprising gCypA were concentrated to approximately 0.5–1.5 mM with 95% H₂O/5% D₂O for subsequent NMR analysis.

NMR spectroscopy

¹H, ¹³C, and ¹⁵N resonance were assigned at 25 °C using a combination of 2D and 3D experiments collected almost entirely on a Varian 600 MHz spectrometer acquired using a ¹³C, ¹⁵N and a ¹⁵N-labeled sample (for review and primary references see (Sattler, Schleucher et al. 1999)). Backbone sequential assignments included a CBCA(CO)NH, HNCACB, HNCO, 3D ¹⁵N-edited and ¹³C-edited NOESY-HSQC experiments (both NOESY experiments employed a $t_{\text{mix}} = 150$ ms). Aliphatic assignments were made using an HCCH-TOCSY, a TOCSY-HSQC ($t_{\text{mix}} = 60$ ms), a 3D HNHA and a 3D HNHB. Due to significant, specific interactions between DSS and gCypA, all spectra were referenced to the HDO resonance. Spectra were processed using NMRPipe (Delaglio, Grzesiek et al. 1995) and all data were analyzed using CCPNmr software (Vranken, Boucher et al. 2005).

Circular dichroism (CD) thermal denaturation

Samples were prepared at 38 (100) μM for hCypA (gCypA) in 50 mM Na₂HPO₄, pH 6.5. CD readings were measured from 30–70(95) °C at 1 °C/min for hCypA(gCypA) at 222 nm on a Jasco J-810 spectropolarimeter. To obtain mid-point values, data were least-squares fit to a variable slope sigmoidal curve.

Results and discussion

Assignments and data deposition

Nearly complete assignments were obtained for both the backbone and side chain resonances of gCypA at 25 °C. All backbone atoms were assigned excluding the backbone amide N of the 9 Pro residues and the C-terminal Pro CA and CB, which culminates in 100% amide proteins, over 99% amide nitrogens, 99% CAs and CBs. The extent of side chain proton assignments was 91%. The ¹H, ¹⁵N, and ¹³C assignments have been deposited at the Biological Magnetic Resonance Data Bank (BioMagResBank, <http://www.bmrb.wisc.edu>) under the accession number 18628.

Secondary structure of gCypA and comparison to hCypA

The probable secondary structure of gCypA was calculated and a comparative analysis to hCypA was conducted (Ottiger, Zerbe et al. 1997). Specifically, the CA chemical shift differences relative to a random coil are shown using the Random Coil Index (RCI) along with the RCI predicted secondary structure (Figure 1) (Berjanskii and Wishart 2005). For

hCypA, both the previously published CA and CB chemical shifts were used (Ottiger, Zerbe et al. 1997) and for gCypA, all nuclear chemical shifts determined here were used. While the majority of predicted secondary structure elements are similar between the two proteins, the predicted β -strand of gCypA comprising residues 68–71 and the predicted α -helix comprising residues 126–129 are somewhat shifted in sequence from their hCypA counterparts. This may indicate that the sequence alignment does not necessarily represent the structural alignment, which is currently being probed with structural studies.

It is important to recognize that the chemical shift predictions of secondary structural elements for hCypA are somewhat different than the actual structures determined by both NMR and X-ray crystallography (see for example PDB accession number 1OCA, 1RMH, 3CYS) and are, therefore, likely significantly different for gCypA as well. The underlying reason for such discrepancies in secondary structure predictions solely using chemical shifts may be that there is a significantly higher representation of aromatic residues in both cyclophilins relative to other proteins (i.e., His, Trp, Phe, and Tyr). These include 20 aromatic residues of the 146 total for gCypA (14%) and 22 aromatic residues of the 165 total for hCypA (13%). Specifically, for the previously determined structures of hCypA, observed β -strands comprise 5–12, 15–24, 53–57, 61–64, 97–100, 112–115, 127–134, 156–163 and α -helices comprise 30–41, 120–122, and 136–145, which all differ by at least several residues from predictions based on chemical shifts alone (in Fig. 1). In fact, for hCypA the critical 3₁₀-helix of residues 120–122 (sequence, EWL) and a relatively long β -strand of residues 127–134 (sequence VVFGKVKKE) are not even identified by RCI. The converse is also true for hCypA where two chemical shift predicted β -strands are found within relatively flexible loops as indicated by relaxation rates (Otting, Ruckert et al. 2000; Eisenmesser, Bosco et al. 2002). For gCypA, several β -strands likely exist that are not identified by chemical shift predictions alone. For example, one likely β -strand is within the active site and includes the highly conserved “catalytic” Arg47 in gCypA (Arg55 in hCypA), which is responsible for destabilization of the prolyl-peptide bond of substrates. For gCypA this β -strand includes residues 45–49 (sequence FHRVI) that are highly conserved relative to hCypA residues 53–57 (sequence FHRII) and is therefore likely present despite not being predicted by the RCI.

It is also important to note that gCypA is monomeric like hCypA and does not self-associate unlike a subclass of other cyclophilins that do form higher order oligomers (Thai, Renesto et al. 2008). This is supported by the fact that gCypA migrated on a Superose-75 as a monomer and, furthermore, no concentration-dependent chemical shift changes were detected within the millimolar concentrations range used for NMR (data not shown). Thus, these data also indicate that gCypA neither weakly nor non-specifically self-associates to higher order oligomers under the conditions required for NMR solution studies. Confirming this absence of aggregation for gCypA that has previously been shown for hCypA is critical to proceed with any future NMR-based dynamic studies (Eisenmesser, Millet et al. 2005), since non-specific self-association could potentially confuse any monitored exchange arising from intramolecular interactions with that arising from oligomerization.

The activity and thermostability of gCypA

The purification of gCypA described here leads to a catalytically active peptidyl-prolyl isomerase (Fig. 2A). To assess activity, ZZ-exchange spectroscopy was utilized since this technique offers a particular sensitive means of assessing the reversible enzymatic activity of cyclophilins as we have previously described (Schlegel, Redzic et al. 2009). Briefly, we generated a recombinantly ^{15}N -labelled model substrate that we have recently shown to be efficiently catalyzed by hCypA (Bahmed, Henry et al. 2012). In the absence of an active peptidyl-prolyl isomerase, this substrate exists in both the cis and trans conformation in solution due to the slow interconversion of the central Pro residue (Fig. 2A, left). However, in the presence catalytic concentrations of gCypA, the appearance of “exchange peaks” indicates that the inherent cis/trans interconversion of the substrate is significantly enhanced (Fig. 2A, right). Therefore, gCypA is indeed an active peptidyl-prolyl-isomerase as predicted by the conserved catalytic Arg47 residue (Fig. 1).

To test the integrity of the gCypA structure with temperature, ^{15}N -HSQC spectra were collected over a wide range of temperatures from 0–40°C (Fig. 2B). As indicated by the well-dispersed spectra, gCypA is well folded over this temperature range. gCypA maintains its structure, with no alteration to its ^{15}N -HSQC spectrum, for at least one week at 40°C, in contrast to hCypA that forms a visible precipitate within hours under the same conditions (data not shown).

To further probe the stability of gCypA over a wider temperature range, both hCypA and gCypA were subjected to circular dichroism (CD) thermal denaturation. While hCypA exhibits a T_m of 51.3 °C, and is fully denatured by 55 °C, the only measured alteration to gCypA's secondary structure occurs with a substantially higher transition mid-point of 67.9 °C (Fig. 2C). Interestingly, even after this transition, gCypA maintains ~70% of ellipticity of the fully folded form, suggesting that significant secondary structure remains present up to at least 95 °C. These studies confirm that gCypA is highly thermostable, an important facet of the work conducted here, providing the impetus for further dynamics studies of gCypA over a much broader temperatures range than is possible for hCypA.

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Figure 1.

The secondary structure elements of gCypA are similar to those of hCypA. Comparisons between gCypA (red) and hCypA (black) are shown using a sequence alignment (Needleman-Wunsch algorithm). RCI values based on the chemical shifts are shown above as β -strands (arrows) and α -helices (barrels) along with the CA shift deviations from random coil.

Figure 2.

gCypA is an active peptidyl-prolyl isomerase that is stable over a broad range of temperatures. A). ZZ-exchange spectroscopy of 1.0 mM ^{15}N -labelled GSFGPDLRAGD produced as in Bahmed et al. (Bahmed, Henry et al. 2012) in the absence (left) and the presence (right) of 20 μM gCypA shows the appearance of exchange peaks that indicate rate enhancement mediated by the enzyme. Arrows denote the cis $\rightarrow$ trans and trans $\rightarrow$ cis transfer of magnetization. The peaks corresponding to the cis and trans amide resonances of the substrate Leu7 are shown at a mixing time of 300 ms and spectra were collected on a Varian

720 MHz spectrometer at 10 °C. B) ^{15}N -HSQC spectra of ^{15}N -labeled gCypA show that the enzyme is fully folded over a broad range. Temperatures included 0 °C (navy), 10 °C (sky blue), 20 °C (green), 30 °C (orange), and 40 °C (red), all collected on a Varian 600 MHz spectrometer. For clarity, only 0°C, 20°C and 40°C plots are displayed for the full spectrum. The inset shows several residues exhibiting chemical shift changes, which includes the catalytic Arg47. C) CD thermal denaturation curves for hCypA (red) and gCypA (blue) measured at 222 nm. Solid lines are best fit curves to the data, with midpoints represented by vertical dashed lines.