Journal of Inorganic Biochemistry 140 (2014) 104–110

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Copper-binding properties of the BIR2 and BIR3 domains of the X-linked inhibitor of apoptosis protein

Yiwen Liang, Patrick M. Ewing, Willem J. Laursen, Valerie T. Tripp, Shubha Singh, Kathryn E. Splan *

Department of Chemistry, Macalester College, 1600 Grand Avenue, Saint Paul, MN 55105, United States

ARTICLE INFO

Article history:
Received 6 March 2014
Received in revised form 14 July 2014
Accepted 14 July 2014
Available online 22 July 2014

Keywords:
Cu(I)
Inhibitor of apoptosis protein
Copper homeostasis
Metal binding
Cysteine mutagenesis

ABSTRACT

The X-linked inhibitor of apoptosis protein (XIAP) is a zinc metalloprotein that has recently been implicated in copper homeostasis. XIAP mediates apoptosis via the inhibition of caspase enzymes through multiple baculovirus IAP repeat (BIR) domains, wherein zinc is coordinated by three cysteine amino acids and one histidine amino acid. XIAP binds copper ions directly at one or more unspecified sites, indicating that the protein may function as a copper sensor. We report the copper-binding properties of an XIAP construct containing the BIR2 and BIR3 domains. Absorption and emission spectroscopic measurements show that XIAP exhibits only a low-to-moderate affinity for Cu(II), but a strong affinity for Cu(I). Cu(I) is observed to bind at multiple sites within the BIR2 and BIR3 domains, including the CXXC motifs of the zinc structural sites and multiple BIR2 surface sites. Mutagenesis-based experiments reveal that surface cysteine residues mediate binding in the BIR2 domain and induce protein oligomerization under elevated copper concentrations. These results constitute the first spectroscopic evidence of copper–XIAP interactions.

1. Introduction

The zinc-metalloprotein X-linked inhibitor of apoptosis protein (XIAP) is a key regulator of programmed cell death, or apoptosis, via inhibition of the caspase enzymes that are the final effectors of apoptosis [1–3]. XIAP contains three zinc-binding, baculovirus IAP repeat (BIR) domains of approximately 70 amino acids and a C-terminal, zinc-binding RING (*R*eally *I*nteresting *N*ew *G*ene) finger. The BIR2 domain binds to and inhibits caspases-3 and 7 [4–6], while the BIR3 domain inhibits caspase-9 [7]. The RING finger acts as an E3 ubiquitin ligase and mediates intracellular levels of multiple apoptotic proteins [8,9]. Owing to XIAP over-expression in multiple tumor types, XIAP has become an important target for the development of cancer treatments [10].

In addition to its role as a regulator of apoptosis, XIAP has recently been implicated in copper homeostasis [11–14]. Specifically, the RING finger of XIAP promotes the ubiquitination and degradation the protein COMMD1, a copper-binding protein that promotes copper export [15,16]. Cellular XIAP levels also decrease in the presence of elevated copper concentrations, resulting in a feedback loop wherein increasing copper concentrations correlate with greater COMMD1 expression [13,14]. Moreover, XIAP binds copper ions directly at multiple unspecified sites via cysteine residues at the BIR and RING domains, leading to a change in electrophoretic mobility and indicating that the protein may function as a copper sensor [13]. A recent report also shows that XIAP interacts with the copper chaperone for superoxide dismutase (CCS) via its BIR3 domain when both proteins are expressed in yeast cells, providing a putative mechanism for XIAP–copper loading [11].

The molecular mechanism by which XIAP binds copper has not been determined. Since a clear role for COMMD1 regulation has been assigned to the zinc-binding RING domain of XIAP, competition between copper and zinc for cysteine residues in this domain may constitute a potential mechanism for XIAP-mediated copper homeostasis. Similarly, copper may compete with zinc bound within the BIR domains, resulting in an unproductive protein conformation and consistent with the observation that copper interferes with the ability of XIAP to inhibit caspase-3 [13]. Accordant with these hypotheses, our lab has recently shown that ~25 residue zinc finger peptides are highly susceptible to displacement of bound Zn(II) by Cu(I) [17]. Finally, XIAP also possesses multiple surface accessible cysteine residues distinct from the Zn(II) binding sites that may mediate the copper interaction (Fig. 1).

In this work we describe the first spectroscopic characterization of the copper binding properties of XIAP. Given the importance of the BIR3 domain in mediating contact with CCS and the observation that copper impacts caspase inhibition mediated by BIR2, we have chosen to base our initial studies on a construct of XIAP containing the BIR2 and BIR3 domains (residues 152–353), referred to as BIR23 throughout this work. This construct contains a total of nine Cys residues, three of which do not participate in Zn(II) interactions: Cys202, Cys213, and Cys351 (Fig. 1). Moreover, elimination of the RING finger allows for the impact of copper on the structure and function of this critical domain to be addressed separately. Specifically, we demonstrate that surface accessible cysteine residues away from the zinc-binding site of BIR2

* Corresponding author. Tel.: +1 651 696 6109; fax: +1 651 696 6432.
E-mail address: splank@macalester.edu (K.E. Splan).

http://dx.doi.org/10.1016/j.jinorgbio.2014.07.010
0162-0134/

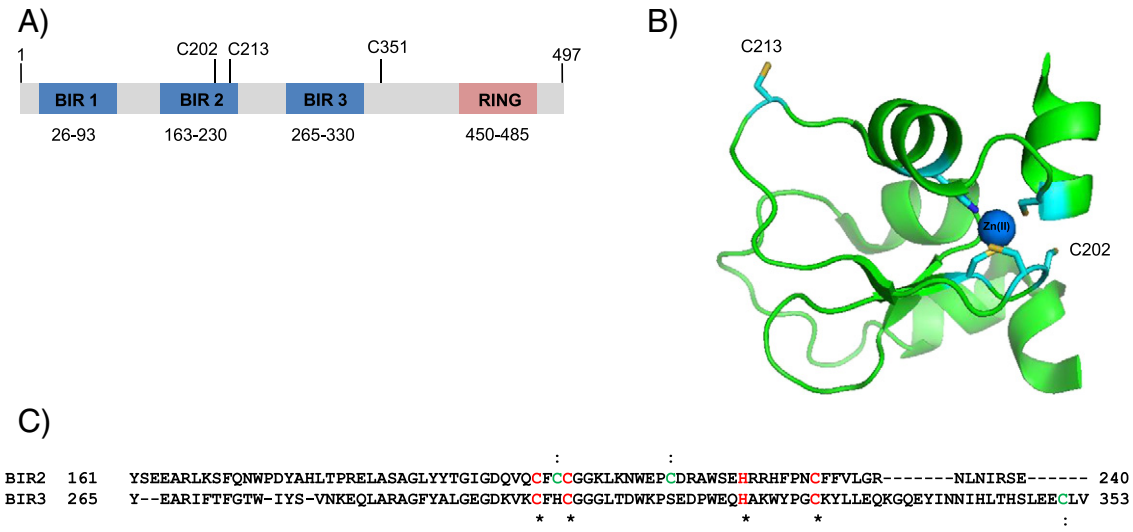

Fig. 1. A.) Domain structure of XIAP, showing the location of all mutations made in this work. B.) Ribbon diagram generated from PDB 4J3Y of the BIR2 domain of XIAP. The residues comprising the CCHC zinc binding site along with two of the cysteine residues mutated in this work are shown. The crystal structure contained the C202A and C213G mutations, and in this schematic, the cysteine residues were modeled back in using PyMol. C.) Sequence alignment of the BIR2 and BIR3 domains of XIAP. Known zinc binding residues are marked in red and with (*). The residues mutated in this study are marked in green and with (:).

constitute high-affinity Cu(I) binding sites. Moreover, we demonstrate that the presence of Cu(I) results in protein oligomerization under elevated copper concentrations, which likely impacts the function of the protein.

2. Material and methods

2.1. Expression, purification, and characterization of XIAP proteins

The plasmid for full-length human XIAP was generously provided by Colin Duckett, University of Michigan. The region corresponding to amino acids 152–353 was amplified by PCR and cloned into pGEX-4T-1 (GE Healthcare Life Sciences) between the BamHI and NotI restriction sites. A PCR-based quick-change mutagenesis method was used to generate all mutations studied herein. All plasmids were confirmed via DNA sequencing.

The resulting plasmids encoding for glutathione *S*-transferase (GST)-fusions of BIR23 variants were transformed into *Escherichia coli* BL21(DE3), grown to mid-log phase at 37 °C in the presence of 0.1 mM $ZnSO_4$, induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and then grown overnight at room temperature. For a 2 L culture, harvested cells were resuspended in sonication buffer (50 mM Tris pH 7.5, 100 mM NaCl) supplemented with PMSF and protease inhibitor tablets (Roche Applied Science) and lysed via sonication. The lysate was passed over glutathione sepharose 4B resin (2 mL; GE Healthcare Life Sciences) and washed with phosphate-buffered saline. The proteins were cleaved from the resin via treatment with thrombin (GE Healthcare Life Sciences) per manufacturer's protocol to yield the desired proteins with the GST tag removed.

Protein concentrations for all constructs were determined via absorbance measurements at 280 nm ($\varepsilon_{280\ nm} = 54{,}890\ M^{-1}\ cm^{-1}$; calculated as described in the literature [18]). Free thiol content was determined upon reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to yield TNB^{2-} ($\varepsilon_{412\ nm} = 14{,}150\ M^{-1}\ cm^{-1}$) [19]. The number of Zn(II) ions per protein upon purification was determined spectroscopically with the reagent 4-(2-pyridylazo)resorcinol (PAR) via a procedure modified from the literature [20]. Briefly, BIR23 samples (5–15 μM) were incubated for 2 h in the presence of PAR (0.5 mM), iodoacetamide (0.9 mM) and guanidine-HCl (4 M) in 100 mM Hepes pH 7.5. [Zn(II)] was determined via absorption at 500 nm utilizing a calibration curve constructed from a Zn(II) atomic absorption standard (15.29 mM in 2% HNO_3) under the same conditions.

2.2. Spectroscopic measurements

Measurements involving Cu(I) were conducted in an anaerobic chamber (Coy Laboratory Products) maintained with 95% N_2/5% H_2 or with gastight syringes and septum-sealed cuvettes prepared anaerobically, unless otherwise noted. Cu(I) stock solutions were prepared anaerobically by dissolving CuCl in 0.01 M HCl/1.0 M NaCl. The Cu(I) concentration was determined spectroscopically by addition of known amounts to excess bicinchoninate (BCA) to form $Cu(BCA)_2$ ($\varepsilon_{562\ nm} = 7900\ M^{-1}\ cm^{-1}$) [21]. All experiments were performed in 200 mM Hepes pH 7.5 and 100 mM NaCl unless otherwise stated.

Absorption spectra were recorded with an Agilent 8453 spectrophotometer (either inside the anaerobic chamber or on the bench top) in either small volume quartz cuvettes (Starna, Inc.) or 1.0 mL disposable cuvettes (BrandTech Scientific, Inc.). To measure Cu(I) binding to BIR23 variants, 10 μM Cu(I) in 1.0 mL and in the presence of specified concentrations of BCA was titrated with 0.1 or 0.2 equivalents of BIR23, and the absorbance at 562 nm was monitored. Samples were allowed to equilibrate between each protein addition until no further spectral changes were observed (~20 min). Care was taken to ensure that the total volume of added protein solution did not exceed 10% of the initial volume, and when absorbance changes were plotted as a function of protein concentration, the concentration of the protein was corrected for dilution. While most trials were performed anaerobically utilizing Cu(I) solutions prepared as described above, identical results were obtained with experiments conducted under ambient conditions utilizing a Cu(II) atomic absorption standard as a copper source and in the presence of 0.2 mM ascorbic acid as a reducing agent. All experiments were performed a minimum of three times.

Fluorescence spectra were recorded with a Varian Cary Eclipse spectrofluorometer. Intrinsic protein fluorescence from BIR23 solutions (1 μM in 600 μL) was measured from 300 to 450 nm upon excitation at 280 nm. 0.4 eq. of either Cu(I) or Cu(II) was added and the fluorescence changes were recorded. All spectral changes were completed within 2–3 min. As an exception, upon addition of Cu(I) at metal:protein ratios of ~3:1, fluorescence intensity decreased slightly over time, possibly owing to small amounts of protein precipitation, although none was visible via the naked eye. Displacement of Zn(II) from