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Conformational selection, dynamic restriction and the hydrophobic effect coupled to stabilization of the BIR3 domain of the human X-linked inhibitor of apoptosis protein by the tetrapeptide AVPI

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ABSTRACT

The XIAP-BIR3 domain blocks a substantial portion of the apoptosis pathway and is an attractive target for novel anticancer agents. The tetrapeptide AVPI, from the protein Smac/DIABLO, binds to the XIAP-BIR3 domain, allowing the cancer cells to die. Here we characterize the binding parameters of AVPI to XIAP-BIR3 and analyze its effects on the thermodynamic stability of this domain. XIAP-BIR3 was exceptionally stable against physical and chemical treatments and became even more stable by interaction with AVPI. Nuclear magnetic resonance experiments demonstrated that conformational selection is taking place upon AVPI interaction with XIAP-BIR3. Molecular dynamics simulations corroborate that the flexibility of XIAP-BIR3 is significantly reduced. The positive binding entropy associated with a loss of conformational entropy involved in the binding indicates that hydrophobic interactions play an important role in the interaction and domain stabilization. The mechanism of XIAP-BIR3 stabilization and its implications for drug affinity optimization are discussed.

1. Introduction

Apoptosis is a process essential for the development and homeostasis of multicellular organisms, and it can lead to a variety of diseases, such as neurodegenerative disorders and cancer, when deregulated [1–3]. Inhibitors of apoptosis proteins (IAPs) are members of an important class of endogenous proteins that can inhibit apoptosis in both intrinsic and extrinsic pathways [4,5]. Among the IAPs that have been studied, the human X-linked IAP (XIAP) appears to be the most potent, and it plays a key role in binding to and inhibiting an initiator caspase (caspase-9) and two effector caspases (caspase-3 and caspase-7) [6–9].

All members of the IAP family present at least one baculoviral IAP repeat (BIR) motif, although many of them contain three motifs. XIAP is a single protein that has three BIR domains and a C-terminal RING finger that are specific for different caspases [10]. Analyses of the structure and function of XIAP have demonstrated that the third BIR domain (BIR3) inhibits caspase-9 selectively and that the inhibition of caspase-3 and 7 is mediated by the linker region between BIR1 and BIR2 [6,7,9,11,12]. The interaction between XIAP and the caspases can be inhibited by Smac/DIABLO, a second mitochondrial activator of the caspases/direct IAP-binding protein, which is released from mitochondria upon initiation of the apoptotic signaling process [13–16]. Structural and biological studies have shown that the interaction between XIAP and Smac/DIABLO takes place between a well-defined surface groove in the BIR3 domain of XIAP and four amino acid residues (AVPI, i.e., Ala-Val-Pro-Ile) at the N-terminus of Smac/DIABLO [14,15]. This four-residue binding motif (i.e., the IAP-binding motif, IBM), is present both in human and mouse caspase-9 [17].

Excessive expression of XIAP and other IAPs occurs in several types of human cancer cells [18–20] and predicts a worse prognosis [21]. Several studies have demonstrated that XIAP plays a critical role in the resistance of cancer cells to chemotherapeutic agents, radiation, and other treatments [22–24]. Thus, different approaches to the inhibition of the antiapoptotic function of XIAP have been explored recently, including the use of antisense oligonucleotides [25] and XIAP inhibitors [26,27]. The tetrapeptide AVPI (from Smac/DIABLO) and other synthetic Smac peptides are able to interact with the XIAP-BIR3 domain, thereby

Abbreviations: BIR, baculoviral IAP repeat; caspase, cysteinyl aspartate-specific proteinase; CD, circular dichroism; Cyt c, cytochrome c; DIABLO, **D**irect **IA**P **B**inding protein with **LO**w pI; IPTG, isopropylthiogalactoside; LB, Luria–Bertani; Smac, **S**econd **M**itochondria-derived **A**ctivator of **C**aspases; UV, ultraviolet.

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inhibiting XIAP and increasing the sensitivity of cancer cells to chemotherapeutic agents both *in vitro* [28,29] and *in vivo* [28–30]. Therefore, several of these approaches have been undertaken to understand the interaction between XIAP-BIR3 and AVPI. Different techniques, including nuclear magnetic resonance [14], crystallography [15] and fluorescence polarization [15,26], have already provided essential data regarding this interaction. Novel anti-IAP candidate drugs have been designed based on the interaction between XIAP-BIR3 and AVPI [31]. However, the thermodynamic aspects of this interaction have not been studied in depth. Many biological processes depend strictly on the thermodynamic stability of the interacting biomolecules [32–37]. In addition, detailed information of the structural-thermodynamic relationships of protein–ligand interactions is of great benefit to structure-based drug design [35]. A better understanding of the structural, dynamic and thermodynamic contributions involved in the formation of the XIAP-BIR3/AVPI complex can provide some insights for drug affinity optimization.

Here, we investigate the binding of AVPI to the XIAP-BIR3 domain to analyze its effects on the thermodynamic stability of this domain using fluorescence spectroscopy, circular dichroism, and calorimetry. We find that the XIAP-BIR3 domain is highly stabilized against urea and thermal denaturation upon interaction with the tetrapeptide AVPI. Nuclear magnetic resonance and molecular dynamics simulations were also used to assist us in understanding the effects of AVPI on the structure and dynamics of the XIAP-BIR3 domain. We determined the thermodynamic parameters of the association between XIAP-BIR3 and AVPI, such as the enthalpy (ΔH), entropy (ΔS), Gibbs free energy (ΔG) and heat capacity (ΔC_p) changes using isothermal titration calorimetry (ITC). We also calculated the unfolding parameters (ΔG, [$U_{1/2}$], and T_m) of XIAP-BIR3 and verified the changes after binding to AVPI. The molecular mechanisms of XIAP-BIR3 stabilization and the implications for drug design are discussed.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade. Experiments were performed in the standard buffer: 15 mM Tris, pH 7.4, containing 150 mM NaCl and 1 mM DTT.

2.2. Protein preparation

The recombinant XIAP-BIR3 domain (residues 238–358) was overexpressed in *Escherichia coli* strain BL21 (DE3) as C-terminal GST-fusion proteins using the pGEX-2 T vector (Pharmacia) in LB medium at 37 °C. This plasmid containing the XIAP-BIR3 sequence was kindly provided by Dr. Yigong Shi [15]. Expression of the protein was induced with 2 mM IPTG. Five hours after induction, the cells were centrifuged (6000 r.p.m. for 20 min; RPR 9.2 rotor; Beckman) at 4 °C and frozen overnight at −20 °C. After thawing, the cells were resuspended in lysis buffer (100 mM Na_2HPO_4, 2.7 mM Na_2HPO_4, 100 mM NaCl, 2.7 mM KCl and 1 mM DTT, pH 7.4) and sonicated. The cell debris was pelleted by centrifugation (15,000 r.p.m. for 20 min; RPR 20.2 rotor; Beckman). The supernatant was applied to a GST affinity column and washed with binding buffer, and the protein was eluted with 10 mM reduced glutathione. After purification, the GST tag was removed using thrombin, and this was followed by a second round of purification using the same column. The purity of the product was confirmed using high-performance liquid chromatography (Superdex-75 column from Pharmacia, data not shown). The samples were dialyzed against the standard buffer (15 mM Tris–HCl, 150 mM NaCl and 1 mM DTT, pH 7.4).

2.3. High pressure and fluorescence spectroscopy

The high-pressure vessel that we used has been described by Paladini and Weber and was purchased from ISS Inc. (Champaign, IL). Fluorescence emission measurements were recorded using an ISSK2 spectrofluorometer (ISS Inc., Champaign, IL). The intrinsic fluorescence was excited at 280 nm, and the emission was observed from 300 to 420 nm. Changes in the fluorescence spectra at pressure p were evaluated based on changes in the spectral center of mass, $\langle \upsilon_p \rangle$:

$$\langle \upsilon_p \rangle = \Sigma \upsilon_i F_i / \Sigma F_i \quad (1)$$

F_i represents the fluorescence emitted at wavenumber υ_i, and the summation was carried out over the range of appreciable values of F.

2.4. Chemical denaturation

The samples were incubated with increasing concentrations of urea (0.5 to 9.3 M) and allowed to equilibrate overnight at 25 °C before measuring the ellipticity in the presence and absence of the denaturant. Each experiment was performed at least three times with different protein preparations.

The free energy change can be correlated empirically using the following equation [38]:

$$\Delta G^0_{[u]} = \Delta G^0_{[0M]} - m[\text{Urea}] \quad (2)$$

$\Delta G^0_{[u]}$ is the apparent free energy of unfolding at each [Urea], $\Delta G^0_{[0M]}$ is the free energy of unfolding in the absence of denaturant, and m is the proportionality constant. The parameter $\Delta G^0_{[u]}$ can be described in the equation $\Delta G^0_{[u]} = -RT \ln K_u$, where R is the gas constant, and T is the absolute temperature in Kelvin. The unfolding constant (K_u) at each urea concentration was determined using the equation $K_u = [(\alpha_u)/(1-\alpha_u)]$, where the values of α_u correspond the fraction of unfolded protein [39].

2.5. Circular dichroism spectroscopy (CD)

CD spectra were recorded using a Jasco J-715 1505 spectropolarimeter. The XIAP-BIR3 samples were diluted to a final concentration of the standard buffer. The spectra were obtained using a 0.01-cm path length quartz cuvette. The spectra were averaged from three scans that were recorded at 50 nm/min and are representative of three independent experiments. The CD spectra of the buffer or AVPI in buffer were subtracted from the respective raw data. Only the far UV region (190 to 260 nm) was analyzed.

2.6. Isothermal titration calorimetry (ITC)

ITC measurements were performed using a VP-ITC calorimeter from MicroCal, Llc. (Northampton, MA). The titration of 10 μM BIR3 with AVPI involved 18 injections (10 × 3 μL and 8 × 7.5 μL) of 0.933 M AVPI solution at 5-min intervals with constant stirring at 400 rpm. The temperature was set at 10, 25 or 37 °C. The BIR3 solutions were degassed under vacuum before the titrations, and the reference cell was filled with Milli-Q water. The heat of dilution of AVPI into the buffer was subtracted from the raw data obtained using XIAP-BIR3. The data were analyzed using the Origin 7.0 software package provided by the manufacturer. The data from the first injection (1 μL) in each experiment was not used in the analysis. Four independent experiments were analyzed separately, and the fitted parameters were averaged. The heat capacity change (ΔC_p) associated with the binding of AVPI to XIAP-BIR3 was obtained from the temperature dependence of the calorimetric enthalpy (ΔH^{cal}) of binding, assuming linear behavior.