The Proapoptotic Protein Smac/DIABLO Dimer Has the Highest Stability As Measured by Pressure and Urea Denaturation[†]

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ABSTRACT: Apoptosis is an essential mechanism of cell death required for normal development and homeostasis of all multicellular organisms. Smac/DIABLO is a dimeric protein important in the control of apoptosis by removing the inhibitory activity of IAPs (inhibitor of apoptosis proteins). *In vitro* studies reveal that dimerization is required for its function. Here we investigate the structural and thermodynamic features of folding and dimerization of Smac/DIABLO. To disturb the folded, dimeric structure, we used high hydrostatic pressure, low and high temperatures, and chemical denaturing agents. Conformational changes were monitored using spectroscopic techniques such as fluorescence and circular dichroism (CD) as well as gel filtration chromatography. Our data show that Smac/DIABLO is very stable under pressures up to 3.1 kbar, even at subzero temperatures. A complete denaturation/dissociation process is obtained when we use high concentrations of urea, which affect its secondary structure as assessed by CD. The association of pressure and subdenaturing urea concentrations also results in complete denaturation/dissociation of the protein. Under these conditions, unfolding of the protein shows concentration dependence that is in accordance with the dimer−monomer dissociation equilibrium, confirming Smac/DIABLO dissociation. These results suggest that most of the treatments lead to a reversible disruption of the dimeric structure with a dissociation constant (K_d) of 34×10^{-21} M (34 zM). This tight dimer is biologically relevant, considering that monomeric mutants bind IAP with low affinity. The extremely high stability of the dimeric form of Smac/DIABLO also implies that once expressed in the cell the protein has a low probability of dissociation and, consequently, loss of function. In addition, the stability in the zeptomolar range is the highest so far measured for a dimeric protein. It also indicates that under most circumstances Smac/DIABLO does not exist as a monomer in the cell and suggests that the dimer-to-monomer equilibrium does not play a regulatory role in the Smac/DIABLO−IAP interaction.

The recent advances in cell and molecular biology have allowed us to understand in more detail the mechanisms that maintain cell viability or induce cell death. Apoptosis plays an essential role in the development and homeostasis of all multicellular organisms (*1, 2*). Deregulation of apoptosis leads to a variety of human pathologies including cancer, autoimmune diseases, and neurodegenerative disorders such as Alzheimer's disease (*3, 4*). The molecular hallmark of apoptosis is the activation of caspases (*5, 6*). Procaspase-9 interacts with cytochrome *c* (cyt *c*)[1] and APAF-1 to form the apoptosome and induce autocatalytic processing of procaspase-9. The mature caspase-9 then activates caspase-3 and -7, leading to the attack of cellular targets and, consequently, the death of the cell. IAPs (inhibitor apoptosis proteins) (*7, 8*) constitute an important class of endogenous cellular inhibitors of apoptosis that prevent the activation of procaspases and inhibit the enzymatic activity of mature caspases (*9–11*). The members of this class are characterized by containing one or more BIR (baculovirus IAP repeat) domains. The IAP-BIR domains have different functions; BIR2 (second BIR domain) is a potent inhibitor of caspase-3, whereas BIR3 (third BIR domain) is responsible for caspase-9 inhibition (*12, 13*).

Another important regulator of apoptosis is released from mitochondria. This novel apoptogenic protein is called Smac (second mitochondria-derived activator of caspases) (*14*) or DIABLO (direct IAP binding protein with low p*I*) (*15*). This protein promotes the activation of procaspases-9, -7, and -3 by binding to IAPs and removing their inhibitory activity. Smac/DIABLO is synthesized in the cytoplasm as a 239 amino acid precursor protein, with the N-terminal 55 amino acids serving as a mitochondrial-targeting sequence (MTS)

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[1] Abbreviations: caspase, cysteinyl aspartate-specific proteinase; CD, circular dichroism; cyt *c*, cytochrome *c*; DIABLO, *d*irect *IAP* *b*inding protein with *lo*w p*I*; HPLC, high-performance liquid chromatography; IAP, inhibitor apoptosis proteins; LB, Luria–Bertani; Smac, *s*econd *m*itochondria-derived *a*ctivator of *c*aspases; UV, ultraviolet; WT, wild type.

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(*14*). The mature Smac/DIABLO protein has 184 amino acids per monomer with a molecular mass of 21 kDa. Its 3D structure has shown an arch-shaped dimer with a high hydrodynamic radius. Additionally, its N-terminus presents four residues, Ala-Val-Pro-Ile (AVPI), that play an indispensable role in Smac/DIABLO function (*16*).

Smac dimerization is considered to be an essential process for its proapoptotic activity, since only dimeric mutants maintain its capacity to promote procaspase-3 activation. This capacity is based on Smac/DIABLO dimeric interface-dependent XIAP BIR2 and Smac/DIABLO N-terminal-XIAP BIR3 interactions (*15, 17*). Recent X-ray crystallographic studies showed that the binding of a peptide derived from Smac/DIABLO is able to suppress the function of IAPs (*17*), although wild type and dimeric mutants have better binding efficiency (*16*). It has been shown that dimeric Smac/DIABLO is able to promote 3–5-fold more activation of procaspase-3 than the monomeric form. Smac/DIABLO peptides can promote the activation of procaspase-3, similar to the monomeric form, but only at higher concentrations. This function suggests the possibility for developing a novel antagonist drug that provides a proapoptotic effect (*18, 19*).

Because of its importance in apoptotic processes and its possible role in cancer therapy, it is crucial to understand the structural and thermodynamic features of Smac. For example, Smac monomeric mutants are not able to induce caspase activity and exhibit lower activation of procaspases (*16*). To study the dimer−monomer equilibrium of Smac, we have used high hydrostatic pressure in combination with other physical and chemical treatments. Recent findings have emerged on the use of high pressure to assess intermediate states in the assembly pathways of several viruses, multimeric proteins, and protein−nucleic acid complexes, addressing many questions regarding macromolecular recognition (*20–22*). Interest in characterizing these states lies in the extent to which they can be related to genuine intermediates present in folding and assembly processes (*23*). High pressure can efficiently promote dissociation of both oligomeric proteins (*24*) and viral structures (*20, 21*). It has a unique property in that its perturbation of macromolecular structures in solution depends exclusively on the volume change of the dissociation or denaturation process.

Here, we show that Smac/DIABLO has impressive pressure stability, even at subzero temperatures (−16 °C). Only when denaturing agents, such as urea, are combined with pressure was the dimer completely dissociated and denatured. After removal of the chemical agent, the tryptophan residues returned to their native-folded environment, suggesting a reversible process. The changes induced by urea or by the combination of urea and pressure were dependent on the protein concentration, making it possible to determine the thermodynamic parameters related to the dissociation and unfolding processes. The dissociation constant value obtained is the highest so far reported for a dimer. A stable and highly intertwined dimer sheds light on the function of Smac/DIABLO in trafficking from the mitochondria to the cytosol to exert its inhibition function on IAP.

EXPERIMENTAL PROCEDURES

Chemicals. All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system. Unless otherwise noted, experiments were performed at 20 °C in standard buffer, 25 mM Tris and 150 mM NaCl, at pH 7.5.

Protein Preparation. Wild-type Smac/DIABLO was overexpressed in *Escherichia coli* strain BL21(DE3) as a C-terminally His-tagged protein using a pET-15b vector (Novagen) in LB medium. The soluble fraction of the fusion protein in the *E. coli* lysate was purified over a Sepharose column (GE Healthcare) and further fractionated by anion-exchange (Source 15Q; GE Healthcare) and gel filtration chromatography (Superdex-200; GE Healthcare) (*16*). After purification, the His tag was removed using thrombin (*25*).

High Pressure and Spectroscopy. The high-pressure vessel has been described by Paladini and Weber (*26*) and was purchased from ISS Inc. (Champaign, IL). The vessel was held at different temperatures with the use of a water circulator bath, using a dry nitrogen gas flush to prevent water condensation on the optical surfaces when we decreased the temperature. Fluorescence emission measurements were recorded on an ISSK2 spectrofluorometer (ISS Inc., Champaign, IL). The tryptophan residues were excited at 280 nm, and emission was observed between 300 and 420 nm. Changes in fluorescence spectra at pressure p were evaluated by changes in the spectral center of mass, $\langle \upsilon_p \rangle$:

$$\langle \upsilon_{\mathrm{p}} \rangle = \sum \upsilon_i F_i / \sum F_i \qquad (1)$$

where F_i stands for the fluorescence emitted at wavenumber υ_i and the summation is carried out over the range of appreciable values of F. Spectral shifts because of the pressure effect on Smac/DIABLO structure at 5.5 M urea were used to follow the dissociation process by converting them into the fraction of dissociated protein at each pressure (α).

The standard volume change of dissociation ΔV and the equilibrium dissociation constant K_d for pressure dissociation extrapolated to atmospheric pressure were determined from the thermodynamic relation:

$$\ln[(\alpha_p)^2/(1 - \alpha_p)] = \ln(K_d/4C) - p(\Delta V/RT)$$

This equation permits the calculation of the standard volume change ΔV from measurements using a fixed protein concentration C at different pressures (*20, 24*).

Light Scattering. Light scattering measurements were carried out in an ISSK2 spectrofluorometer (ISS Inc., Champaign, IL). Scattered light was collected at an angle of 90° to the incident light. The samples were excited at 320 nm, and the scattered light was collected at the same wavelength. These measurements were carried out during all experiments in order to follow possible aggregation processes. Data are not shown since no aggregation was observed.

Chemical Denaturation. The samples were incubated with increasing concentrations of urea (1−10 M) and guanidine hydrochloride (data not shown) and allowed to equilibrate overnight prior to performing measurements. Measurements were done in the presence and absence of the denaturant agent. Urea denaturation experiments were performed by incubation of the samples in each concentration of agent overnight, followed by spectroscopic measurements. Each experiment was performed at least three times with different protein preparations.

The free energy change can be correlated empirically using the equation (*27*):

$$\Delta G_u = \Delta G^o{}_d - m[\text{urea}] \quad (2)$$

where ΔG_u is the free energy of denaturation at each [urea], $\Delta G^o{}_d$ is the free energy of denaturation in the absence of denaturant, and m is the proportionality constant. The free energy of dissociation was calculated using the Gibbs equation ($\Delta G = -RT \ln K$), and the dissociation constant at each urea concentration (K_u) was determined from the values of α_u (*28*). The integrated expression is

$$\ln[(\alpha_u)^2/(1-\alpha_u)] = [\text{urea}](m/RT) + \ln(K_d/4C) \quad (3)$$

The $\Delta[U]_{1/2}$ was calculated using the equation:

$$\Delta[U]_{1/2} = (RT/m)\ln(C_2/C_1) \quad (4)$$

Circular Dichroism Spectroscopy. CD spectra were obtained in a Jasco spectropolarimeter, model J-715 1505. The spectra were obtained in 25 mM Tris and 150 mM NaCl, pH 7.5, buffer using a 0.2 cm path-length quartz cell. Spectra were the average of two scans at a 50 nm/min speed. Only the far-UV region from 190 to 300 nm was analyzed.

High-Performance Liquid Chromatography. High-performance liquid chromatography (HPLC) was performed in a Shimadzu system using a prepackaged Superdex-200 column (Pharmacia). The system was equilibrated in 25 mM Tris and 150 mM NaCl, pH 7.5, buffer. A flow rate of 0.7 mL/min was used. Elution of samples was monitored by tryptophan fluorescence emission at 325 nm (excitation at 280 nm) and absorbance at 260 nm (data not shown).

RESULTS

During apoptosis, IAP-mediated caspase inhibition is abrogated by the mitochondrial protein Smac/DIABLO. Upon apoptosis stimuli, Smac/DIABLO is released from the intermembrane space of mitochondria into the cytosol, together with cyt *c*, and interacts with multiple IAPs and removes IAP-mediated inhibition of both initiator and effector caspases (*9, 11, 16*). Since the discovery of Smac/DIABLO is recent, several questions about this protein and gaps in its mechanism of action need to be answered. Furthermore, many studies focused on the development of drugs that bind to IAPs, promoting its inhibition, have been performed. Since the 3D structure of the BIR3 IAP and Smac/DIABLO complex is available, it becomes necessary to understand the dynamics and stability of this complex to provide new elements for facilitating the design of new drugs.

In this work, our intention was to characterize the stability of Smac/DIABLO and obtain some insights about the monomer–monomer interaction that plays an essential role in Smac/DIABLO−IAPs interactions. With this aim, we have used high pressure, high temperature, subzero temperature, and chemical denaturing agents such as urea to promote perturbations in Smac/DIABLO structure. Conformational changes were monitored by following the changes in the average energy emission of tryptophan fluorescence. This protein contains four tryptophan residues per dimer, two of which are located near the dimerization interface. The dimer has an arch-shaped architecture, which confers a high hydrodynamic radius (Figure 1).

Urea-Induced Denaturation of Smac/DIABLO. Urea is a potent denaturing agent of proteins that acts upon their tertiary structure by disrupting hydrogen bonds and hydrophobic and electrostatic interactions. In order to characterize

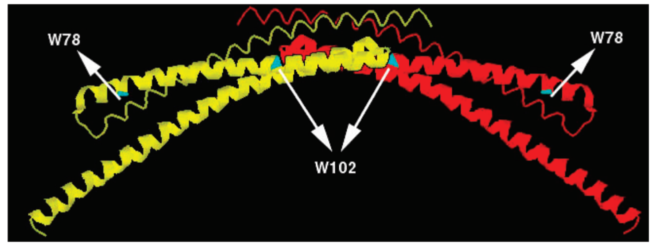

FIGURE 1: Schematic representation of the three-dimensional structure of Smac/DIABLO. Smac/DIABLO is an arch-shaped homodimer comprising 184 amino acids per monomer. There are four tryptophan residues per dimer, two of which are located near the dimerization interface. These residues are very important to our study, since we can follow the structural changes of the protein using fluorescence spectroscopy techniques. PDB ID: 1FEW (*16*).

the dissociation/denaturation of Smac/DIABLO, we performed different measurements using increasing urea (1−10 M) and guanidine hydrochloride (data not shown) concentrations. The samples were incubated overnight in each concentration of urea. We first monitored denaturation by examining changes in the spectral center of mass of the intrinsic tryptophan fluorescence emission and changes in the circular dichroism spectra (ellipticity at 222 nm). Interestingly, changes in the secondary and tertiary structure overlap (Figure 2). Significant changes in the center of mass values and ellipticity at 222 nm were only observed above 6 M urea (Figure 2A). The data clearly suggest a high stability of Smac/DIABLO under this treatment, since only after incubation with high urea concentrations was the structure affected. Upon converting the data from changes in the center of mass into the degree of dissociation, we obtained a K_d of $\sim 34 \times 10^{-21}$ M (Table 1). This 34 zeptomolar (zM) dissociation constant reflects a huge stability, the highest so far measured for a dimer (*29*) (Table 1). To ascertain whether the spectral changes reflect a dimer–monomer dissociation process, we performed the experiment using two protein concentrations, 50 and 500 μg/mL Smac/DIABLO. The measured $\Delta[U]_{1/2}$ (Table 1) was equal to the theoretical value determined from eq 4 (see Experimental Procedures). Protein concentration dependence on urea-induced denaturation was observed in these measurements, demonstrating that the protein was denaturing and dissociating (Figure 2B).

We performed gel filtration chromatography measurements in order to confirm whether urea is able to disrupt the dimerization interface. By analyzing the elution profile of the protein in the presence of urea (running buffer containing urea), we found that the elution time of the urea-denatured species was about the same as the dimer (Figure 3). This result suggests that monomer forms have an extended conformation, when the running buffer contains urea, that elutes at the same time as the dimer.

Pressure Effects on Smac/DIABLO Stability. Recent studies on the effect of pressure on macromolecular assemblages have provided new information on protein–protein and protein−nucleic acid interactions as well as virus assembly (*21, 22*). The effects of high pressure on Smac/DIABLO stability were also investigated. Hydrostatic pressure induces less drastic changes than urea and makes it possible to detect different states in the dissociation pathway (*21*). This method takes advantage of the fact that it does not chemically

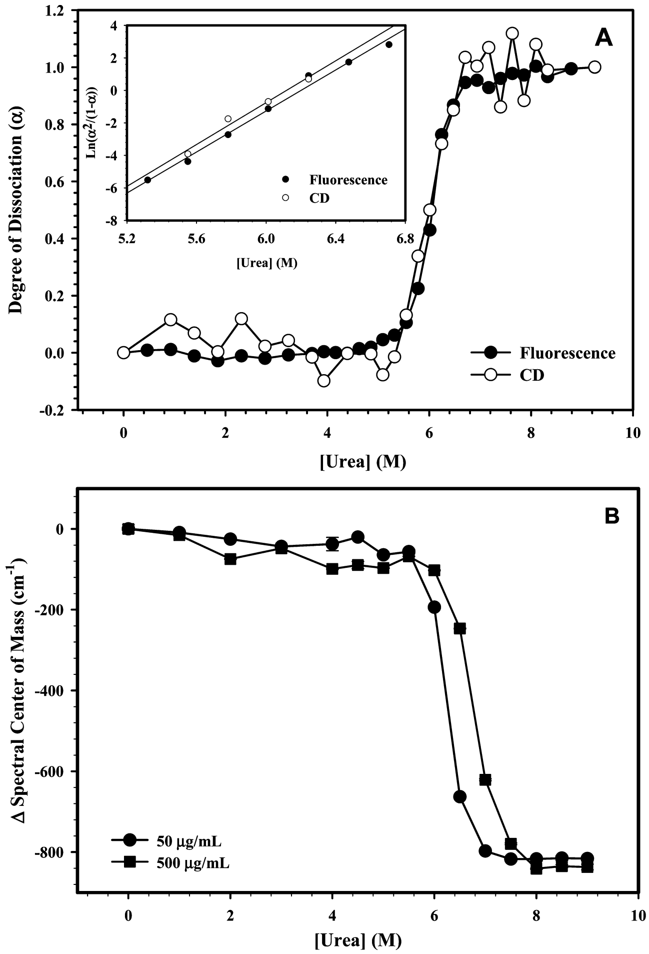

FIGURE 2: Structural changes of the Smac/DIABLO dimer upon dissociation/denaturation by urea. In order to investigate the effects of urea on Smac/DIABLO stability, we utilized increasing concentrations of urea (1−10 M) as shown in the curve above. A synergism process was observed during dissociation/denaturation. Inset: Plot of $\ln[(\alpha_p)^2/(1 - \alpha_p)]$ versus urea concentration. The sample concentration utilized was 200 μg/mL (A). The concentration dependence of the protein under urea treatment was also investigated (B). The sample was excited at 280 nm, and the fluorescence emission was measured at 300−420 nm. The data are representative of three experiments. The samples were incubated overnight at each urea concentration, and the measurements were carried out at 20 °C.

interfere with the sample. Changes occur by modifying the volume of the system. Here, we subjected the samples to pressures ranging from 0.001 to 3.1 kbar. We clearly verified, through changes in the center of mass values, that the protein was not significantly affected, showing a high-pressure stability (Figure 5A). Even after 300 min at 3.1 kbar, no significant changes were observed (data not shown). This result corroborates the high stability of the protein as measured by urea denaturation.

As previously described for some proteins, pressure alone does not cause complete denaturation due to a small volume change or a large stability. In order to reach complete denaturation, it is necessary to combine pressure with temperature (high or low) and/or denaturing agents (*20, 21*).

In this way, we first utilized subzero temperatures in an attempt to promote complete protein denaturation by physical methods. A great number of proteins and viruses exhibit cold denaturation and/or inactivation (*20, 30, 31*). Since at 2.5 kbar the freezing point of water is shifted to approximately −20 °C, we can utilize high pressure to reach subzero temperatures while maintaining an aqueous system in the liquid state. We were not able to observe a significant difference when the protein was subjected to 3.1 kbar at −16 °C (Figure 4B).

Since the previous conditions were not able to cause complete denaturation/dissociation of the protein, we associated pressure and subdenaturing urea concentrations to promote a more drastic disturbance on protein structure. Structural changes were followed by means of the intrinsic fluorescence spectra (since each monomer contains two Trp residues at positions 78 and 102) and CD. High hydrostatic pressure in the presence of different concentrations of urea was applied to Smac/DIABLO, and the results show that a significant shift in the Trp spectrum (corresponding to changes in the spectral center of mass) only became evident in the presence of 5 and 5.5 M urea (Figure 5). The reversibility of the denaturation process induced by pressure and urea was suggested by following the spectral change on decompression. The value of the center of mass for Trp emission returned to values close to the initial value (prior to pressure application) (Figure 5).

Concentration Dependence of the Pressure Effects on Smac/DIABLO. To investigate whether the spectral changes reflect a dimer−monomer dissociation process, we pressurized the sample in the presence of 5 M urea at three different concentrations to test concentration dependence on protein dissociation (Figure 6). The dissociation process was more pronounced as the total protein concentration decreased, whereas secondary and/or tertiary conformations are not affected by this factor (*32*). We observed that the curves were shifted to lower pressures by the decrease in protein concentration, showing concentration dependence characteristic of a dimer−monomer dissociation equilibrium (*20, 24, 32, 33*).

The pressure experiment was also performed in the presence of 6 M urea at two Smac/DIABLO concentrations (200 and 500 μg/mL) and demonstrated complete denaturation/dissociation at high pressure (data not shown). At this urea concentration, even at atmospheric pressure there was already an effect on the Smac/DIABLO structure at 200 μg/mL concentration but not at 500 μg/mL (Figure 2).

Interestingly, for the higher protein concentration (500 μg/mL), the center of mass values almost completely returned when pressure was released. At this concentration, the protein presented a high degree of secondary structure as observed by circular dichroism after pressure release (Figure 7B). With these data, it was clear that the protein recovers its structure when the pressure is released. The protein was less structured at a lower concentration (200 μg/mL), in accordance with the center of mass values (Figure 7A). On the other hand, we observed no differences between the control and the sample by gel filtration chromatography after pressurization in the presence of 6 M urea (Figure 8). In this case, since the dissociation process was evaluated by fluorescence measurements, the results indicate that the pressurized sample in the presence of urea, similar to the sample in the presence of high urea concentrations, dissociates to monomers showing a high hydrodynamic radius ("extended monomers"), similar to the dimer. After decompression, the protein either remains as monomer or the dissociation process is reversed, as suggested by the partial return of the values for the center of mass of Trp after decompression. These

Table 1: Thermodynamic Parameters for Dissociation of Smac/DIABLO[a]

treatment	K_d (zM)	ΔG_d (kcal/mol)	$\Delta[U]_{1/2}$ (M)	m [kcal/(mol·M)]	ΔV (mL/mol)
urea (CM)	34.75 ± 0.38	29.25 ± 0.07	0.361 ± 0.01	3.76 ± 0.03	−56.0 ± 3.5

[a] Values for the center of spectral mass (CM) were determined. The dissociation constant at atmospheric pressure (K_d) was obtained as described under Experimental Procedures (equation). All thermodynamic parameters are those at atmospheric pressure. $\Delta[U]_{1/2}$ corresponds to the shift in the values of urea concentration that caused 50% dimer dissociation when comparing Smac/DIABLO concentrations of 50 and 500 μg/mL. The free energy of dissociation was calculated using the Gibbs equation ($\Delta G_d = -RT \ln K_d$). The temperature was 25 °C.

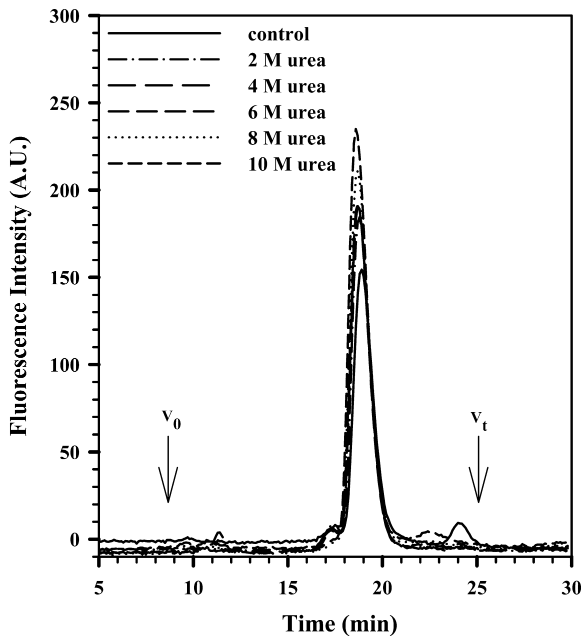

FIGURE 3: HPLC of Smac/DIABLO under urea treatment. High-performance liquid chromatography was performed using a gel filtration column, Superdex 200 (GE Healthcare). The system was equilibrated in 25 mM Tris and 150 mM NaCl, pH 7.5, in the presence of each urea concentration. A flow rate of 0.7 mL/min was used. Sample elution was monitored by tryptophan fluorescence (excitation at 280 nm, emission at 325 nm).

results were confirmed by nondenaturing gel electrophoresis (data not shown).

High-Temperature-Induced Denaturation of Smac/DIABLO. To investigate the effect of high temperature on the Smac/DIABLO dissociation/denaturation, we increased the temperature of the sample to 85 °C and measured the fluorescence emission of tryptophan. There were no significant changes in the center of mass values until 60 °C was reached (even after 280 min incubation), showing that high temperature is only able to cause changes in the tryptophan environment at temperatures beyond this range, in contrast to other proteins that exhibit denaturation at 60 °C (data not shown).

We also investigated whether high temperatures could cause changes in secondary structure. With this aim, we performed CD experiments while the temperature was increased to 85 °C, measuring the raw ellipticity at 222 nm. We found that the loss in secondary structure was only observed at temperatures above 60 °C (Figure 9), in accordance with the changes in tertiary structure analyzed by the fluorescence emission of Trp. The high-temperature-induced denaturation was irreversible, confirming that this is a more drastic process (Figure 9). In all conditions, we checked whether there was aggregation of the protein, as determined by light scattering (data not shown). There was no tendency toward protein aggregation in any of the treatments carried out.

DISCUSSION

Apoptosis is an orderly process utilized for the depletion of dispensable or injured cells and is essential to morphogenesis and the maintenance of homeostasis in an organism (*34*). Several proteins are involved in programmed cell death, promoting or inhibiting apoptosis. This equilibrium is broken in the cancer cells, for example, in which disordered growth is detected. In the intrinsic cell death pathway, the key event leading to the activation of caspases is the release of several proapoptotic proteins from the intermembrane space of mitochondria into the cytosol (*35*). The "second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low p*I*" (Smac/DIABLO) was recently identified as a protein that is released from mitochondria in response to apoptotic stimuli and promotes apoptosis by antagonizing the inhibitor of apoptosis proteins (IAPs) (*14, 15*). Recent studies have shown that the different kinetics of Smac/DIABLO release from the mitochondria when compared to cyt *c* could be explained by the high hydrodynamic radius of the Smac/DIABLO dimer (*36*). The Smac/DIABLO structure and the maintenance of its function after passage through mitochondria membrane could be explained by the high stability of this dimeric structure, as shown in this work.

Here, we describe the high stability of the Smac/DIABLO dimer in response to physical and chemical denaturing agents. The stability observed in the zeptomolar range is the highest so far measured for a dimeric protein or for protein interactions. The closest stabilities described in the literature are reported in the femtomolar affinity range. For example, the LexA repressor dimer bound to cognate DNA with a dissociation constant K_d = 20 fM and a free energy of association ΔG = −18.8 kcal/mol (*33*), ecotin bound to chymotrypsin and trypsin with a K_d = 130 fM and a ΔG = −17.5 kcal/mol (*37*), and the antibody scFv 4 M5.3 bound to antigen with a K_d = 48 fM and a ΔG = −18.2 kcal/mol (*38*).

Smac/DIABLO is ubiquitously expressed in several organs, and its 3D structure was solved in 2000 by Shi and colleagues (*16*). This is a protein of particular interest since it inhibits the inhibitory effect of IAPs, allowing tumorigenic cells to enter apoptosis. A better understanding of its structural aspects is crucial for understanding their biological functions and role in the pathogenesis of several diseases, as well as to aid the development of new strategies to treat, for example, cancer. Recent studies have shown that overexpression of Smac/DIABLO can induce apoptosis and/or sensitize the resistant cancer cells to death receptor- or cytotoxic drug-induced apoptosis. These findings suggest that Smac/DIABLO plays an important role in the regulation of apoptotic responses in cancer cells to both immune- and drug-mediated therapies (*39*). The extremely high stability of the dimeric form of Smac/DIABLO implies that the protein, once

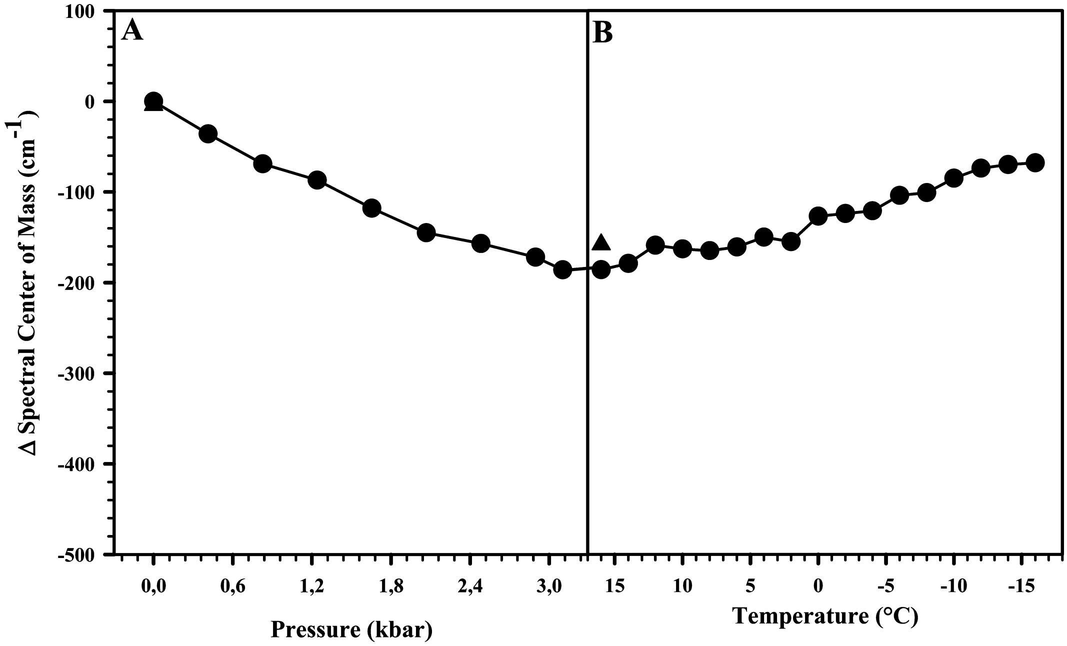

FIGURE 4: Pressure and cold stability of Smac/DIABLO. The effect of pressure on Smac/DIABLO was measured up to 3.1 kbar at room temperature (A) and under pressure (3.1 kbar) combined with subzero temperatures (B). Filled triangles represent the return to room temperature and atmospheric pressure. The incubation time at each pressure was 10 min. Fluorescence data points are the average and standard deviation of three experiments. Other conditions are the same as those presented in Figure 2.

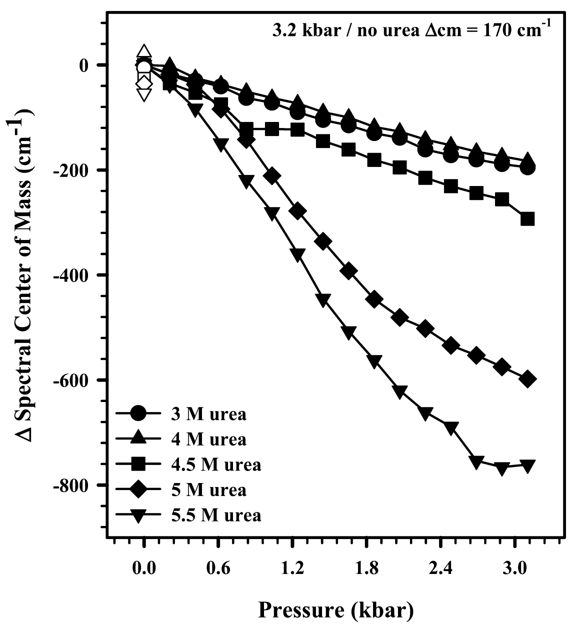

FIGURE 5: Pressure-induced effects on Smac/DIABLO in the presence of subdenaturing urea concentrations. The effect of pressure combined with subdenaturing urea concentrations on Smac/DIABLO (200 μg/mL) dissociation was measured by the spectral center of mass of tryptophan fluorescence emission. Fluorescence data points are the average and standard deviation of three experiments. Other conditions are the same as those presented in Figures 2 and 4. The return values are shown as open symbols for each corresponding concentration. The measurements were carried out at 20 °C.

expressed, looks for its target (IAP) by diffusing into the cytoplasm with little chance of dissociating and losing its function.

We have investigated the high pressure, chemical denaturant, and temperature stability of Smac/DIABLO by using mainly spectroscopic methods. Thus, only high urea concentrations or pressure combined with urea led to a complete exposure of Trp residues, including the residues present in the dimer interface, demonstrating the high stability of the protein (Figures 2 and 5). Furthermore, in the presence of urea, the pressure-dissociation process was concentration-dependent, with $p_{1/2}$ (half-dissociation pressure) shifting to higher pressures as the concentration was increased (Figure 6). Since the dissociation process was shown by fluorescence emission of Trp and concentration dependence, we suggest that the pressurized sample in the presence of urea dissociates to monomers showing a high hydrodynamic radius ("extended monomers"), similar to the dimer. After pressure release at low urea concentrations, the dissociation process is reversible, as suggested by the partial return of the values for the center of mass of Trp after decompression.

The interactions between amino acid residues in the tertiary and quaternary structures of proteins are similar (van der Waals forces and those of electrostatic nature), and similar responses to pressure are therefore expected. Hydrostatic pressure drives the structure of proteins to a thermodynamic state of smaller volume (*20, 24*). Protein folding and protein–protein interactions are normally accompanied by an increase in volume because of the combined effects of the formation of cavities excluding solvent and the release of bound solvent (*20*).

For most dimers, the range of dissociating pressure is generally below 3 kbar (*21*). In this range, the separated chains need not experience further pressure effects, as single-chain proteins are generally stable below 5 kbar (*24*). However, in some cases, dimers behave unexpectedly under pressure. For example, in the case of the coiled-coil dimer of tropomyosin, pressure leads to the formation of a denatured dimer (*40*). The formation of a denatured coiled coil reveals an intermediate with folding subdomains, which is probably important in the folding pathway of this protein. Here we demonstrate that the Smac/DIABLO protein shows a different behavior under pressure treatment, since the range usually sufficient to dissoci-

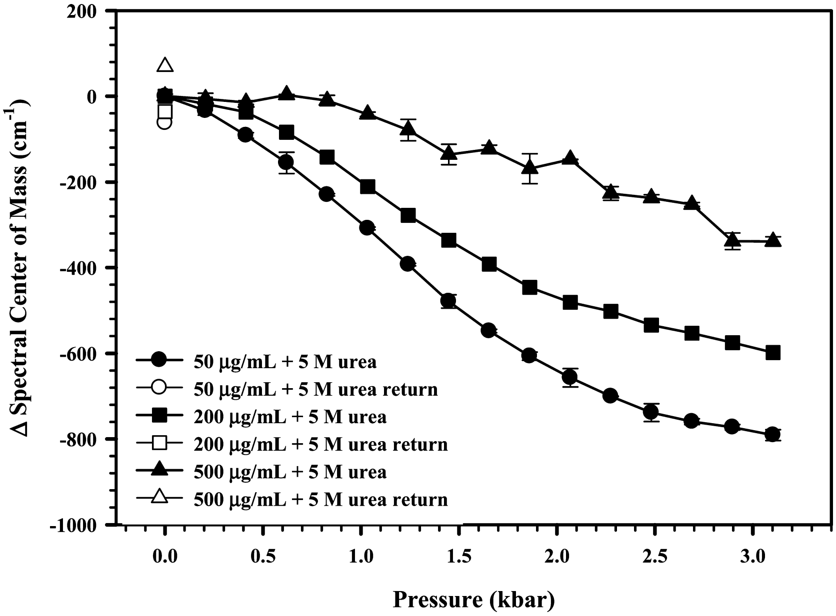

FIGURE 6: Concentration dependence and dissociation of Smac/DIABLO induced by pressure. The effect of pressure in the presence of 5 M urea on Smac/DIABLO dissociation was measured by the center of mass of tryptophan fluorescence emission. The sample concentrations utilized were 50, 200, and 500 μg/mL. The measurements were carried out at 20 °C. Fluorescence data points are the average and standard deviation of three experiments. Other conditions are the same as those presented in Figures 2 and 5.

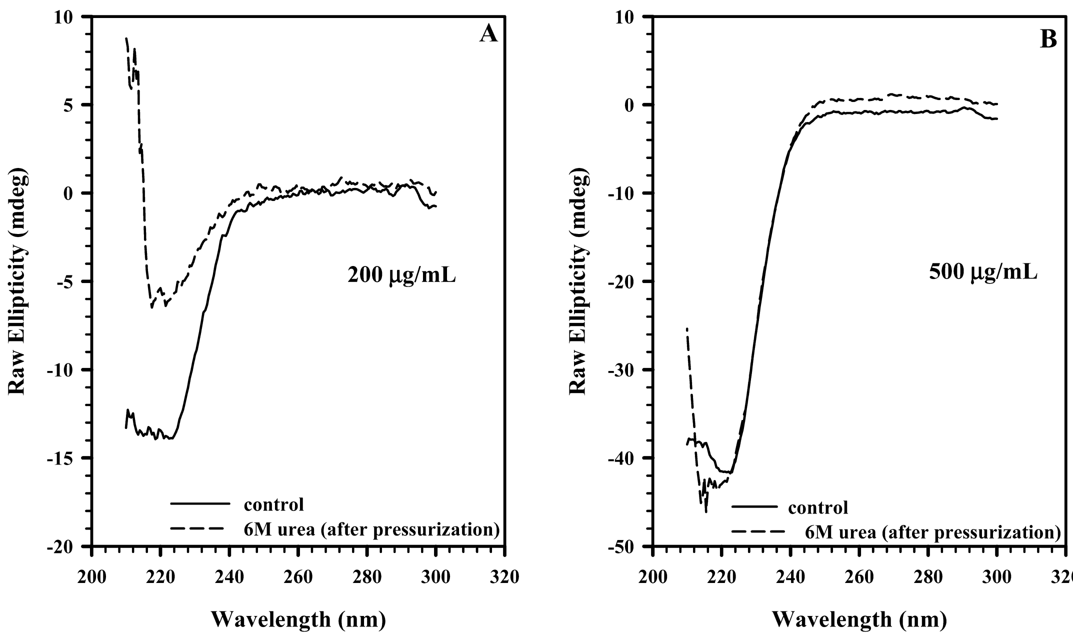

FIGURE 7: Far-UV circular dichroism of Smac/DIABLO after high-pressure treatment. Changes in the α-helix motif of Smac/DIABLO in the presence of 6 M urea and after high pressure were examined. The sample was diluted in standard buffer, 25 mM Tris and 150 mM NaCl (pH 7.5), and measurements were done using a 0.1 cm path-length quartz cell. The sample concentrations utilized were 200 μg/mL (A) and 500 μg/mL (B). The spectropolarimeter used was a Jasco J-715 1505 model. Wavelength range: 300−190 nm. The measurements were carried out at 20 °C. The data are representative of three experiments.

ate globular dimers is not able to promote its dissociation. This can be explained by its conformational structure in a peculiar arched shape that does not retain many cavities during folding and oligomerization, as found in many globular proteins. This conformation may be responsible for its structural stability, since its activity depends on the dimeric state.

The fluorescence data showed that the midpoint of the unfolding transition increased to higher urea concentration with increasing protein concentration (Figure 2B). The monophasic transition curves for fluorescence data and the protein concentration dependence suggest a two-state unfolding/refolding model (*41*). This allowed us to estimate the conformational stability of Smac/DIABLO in the absence of denaturant (ΔG), the dissociation constant (K_d), and the *m*-value per molar urea (Table 1). It is possible to assume two basic models for the equilibrium unfolding/refolding pathway of Smac/DIABLO, as already seen in other works with similar results (*42*). In model 1, the folded dimer (N_2), folded monomer (N), and unfolded monomer (D) are significantly populated at equilibrium:

$$N_2 \Leftrightarrow 2N \Leftrightarrow 2D$$

where $K_1 = [N]_2/[N_2]$ is the equilibrium constant for the bimolecular dissociation reaction and $K_2 = [D]/[N]$ is the

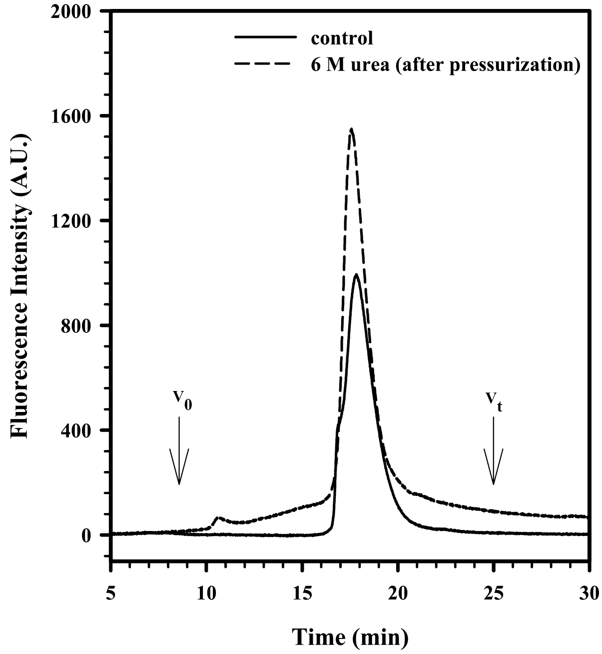

FIGURE 8: HPLC of Smac/DIABLO after pressurization. High-performance liquid chromatography was performed using a gel filtration column, Superdex 200 (GE Healthcare). The system was equilibrated at 25 mM Tris and 150 mM NaCl (pH 7.5). A flow rate of 0.7 mL/min was used. Sample elution was monitored by tryptophan fluorescence (excitation at 280 nm, emission at 325 nm). The data are representative of three experiments.

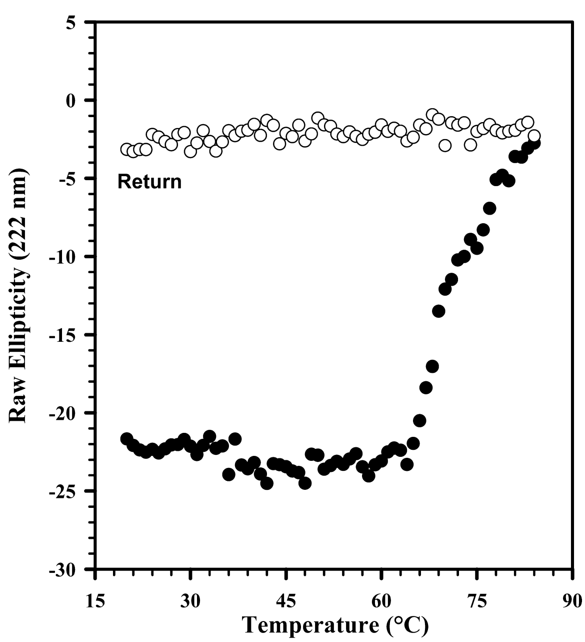

FIGURE 9: Far-UV circular dichroism of Smac/DIABLO under high-temperature treatment. Changes in the secondary structure of Smac/DIABLO using increasing temperatures (●) and the process of return at room temperature (○) were examined. The sample was diluted to a final concentration of 200 μg/mL, and the spectra were obtained in the standard buffer, 25 mM Tris and 150 mM NaCl (pH 7.5), using a 0.1 cm path-length quartz cuvette. The spectropolarimeter used was a Jasco J-715 1505 model. The data are representative of three experiments.

equilibrium constant for the unimolecular unfolding reaction. In this case, K_1 should be significantly larger than K_2, and the quaternary interaction stabilizing the dimeric association will be higher than the secondary and tertiary interactions stabilizing the folded monomer. The folded monomer is

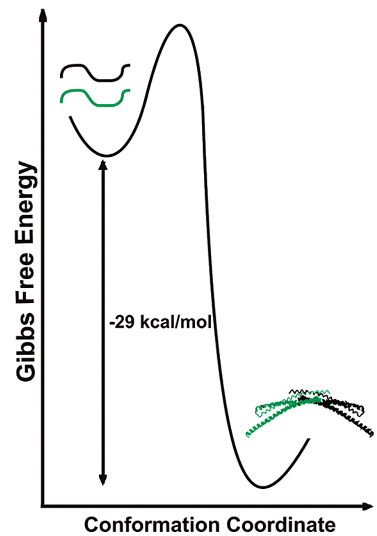

FIGURE 10: Gibbs free energy diagram for Smac/DIABLO dimer folding. Free energy diagram showing the conversion between the unfolding state and the Smac/DIABLO dimer.

assumed to have a tertiary structure similar to that of the dimer subunit but is less active because the dimeric structure is required for its function (*43*).

In model 2, only folded dimer (A) and unfolded monomer (U) are significantly populated:

$$N_2 \Leftrightarrow 2D$$

where $K = [D]^2/[N_2]$ is the equilibrium constant for the bimolecular reaction. The two states are now a native dimer, N_2, and a denatured monomer, D. In this model, the monomer does not exist at significant concentrations at equilibrium because the quaternary interactions are necessary for stabilization of the folded monomeric form. Other papers have shown, by analyzing crystallography data of intramolecular interactions of dimers, that in some proteins, for example, nerve growth factor (*44*) or Trp repressor (*45*), the isolated monomer would not be capable of maintaining its folded conformation. Experimental data presented in this study (Figures 2, 5, and 6), such as protein-concentration dependence and monophasic transitions, support model 2, which describes a highly cooperative and two-state pathway for the equilibrium unfolding/refolding of Smac/DIABLO. Figure 10 shows a simplified energetic diagram to represent the equilibrium between the unfolded monomers and the folded dimer.

To identify unfolded monomers that form at high urea concentrations, such as 8 M urea, we utilized a size exclusion column (Figures 3 and 8) and native gel electrophoresis (data not shown); however, no alteration in the elution profile could be observed. We propose a model in which the Smac/DIABLO protein, when submitted to high urea concentrations or pressurized to values near 3.2 kbar, in the presence of urea, undergoes complete denaturation/dissociation to its monomeric form that, notably, appears as a huge hydrodynamic radius ("extended monomers"). However, these processes are probably reversible, and reassociation of the monomers into dimers occurs after pressure release.

The size of the protein or the amount of its surface area exposed to solvent upon unfolding is the major structural determinant for the *m*-value (*46*). The magnitude of the *m*-value is also indicative of a highly cooperative two-state unfolding/refolding process (*27*). The relatively high value calculated for the volume change (−56 mL/mol) is in accordance with the high *m*-value [3.76 kcal/(mol·M)]. The fact that a folded monomeric state was not detected at any stage indicates that it is thermodynamically unstable. This implies that the subunit interactions of the homodimeric protein are important for stabilizing the tertiary structure of the individual monomers (*29*). Thus, the quaternary interactions are highly favored and necessary for Smac/DIABLO structure and are probably responsible for the zeptomolar affinity observed between the monomers and the exceptional stability of the homodimeric protein.

Other researchers have shown that Smac dimerization increases the stability and affinity of the Smac/XIAP interaction for the BIR2 and BIR3 binding domains (*47*). In addition, mutations that impaired Smac/DIABLO dimerization were associated with its diminished caspase-promoting activity (*16*). Our results strongly suggest that the loss of both Smac/DIABLO activity and its affinity for IAP in the monomeric form is associated with the fact that the monomers are unstable. Thus, it is clear that Smac/DIABLO dimerization plays an important role in the normal function of the cell.

The overexpression achieved for Smac/DIABLO in *E. coli* indicates the foreign protein's ability to fold efficiently to a thermodynamically stable, proteolysis-resistant, and functional conformation in the bacterial intracellular environment. Smac/DIABLO is also able to spontaneously refold to its native form *in vitro* (Figures 5 and 6) by diluting the denaturant or after pressure release, suggesting that chaperone/foldases are not essential for folding/refolding.

With a high unfolding/dissociation free energy of 29.25 kcal/mol, Smac/DIABLO is a fully native dimer at physiological concentrations greater than or equal to 10^{-21} M. The high free energy of Smac/DIABLO unfolding is unusual for a two-state unfolding dimer (Figure 10); the *E. coli* Trp arcorepressor, for example, which is also a homodimeric protein, has a free energy of unfolding of 23 kcal/mol (*48*). The equilibrium constant for the breakage of two monomer contacts is determined by the rate constants of dissociation (K_{diss}) and association (K_{assoc}). The dissociation rate constant for two monomer contacts can be expressed as $K_{diss} = K_{assoc} \exp(\Delta G/RT)$. As a general rule, the differences in the equilibrium constant of protein interactions arise from modifications of the rate constant of dissociation (*49*). If we consider that the association constant has the highest possible value, e.g., limited by diffusion, $K_{assoc} = 10^9\ M^{-1}\ s^{-1}$ (*50*), the estimated value of the dissociation rate constant is 10^{-12}, which gives a half-time of dissociation, $t^{1/2}$, of approximately 20000 years. The closest example of very high stability is the reported interaction for an antigen–antibody complex (*38*). Single-chain antibody mutants have been evolved *in vitro* with an antigen-binding equilibrium dissociation constant of $K_d = 48$ fM and slower dissociation kinetics (half-time >5 days) than those for the streptavidin−biotin complex (*38*). Therefore, under the nonequilibrium conditions inside the cell, a persistency of the Smac in the dimeric form is extremely favored.

In conclusion, we describe the highest stability (zeptomolar range) so far measured for a dimeric protein or for protein interactions and show that this overall stability and strength of the dimeric interface indicate that Smac/DIABLO, under most circumstances, does not exist as a monomer in the cell, confirming that the dimer to monomer equilibrium does not play a regulatory role in the Smac/DIABLO−IAP interaction.

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