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Bcl-x_L Forms Two Distinct Homodimers at Non-ionic Detergents: Implications in the Dimerization of Bcl-2 Family Proteins

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As the key regulator of apoptosis, Bcl-2 family protein controls the cell death by forming homo- or heterodimers among anti-apoptotic and pro-apoptotic members of this family. Here we have studied Bcl-x_L homodimerization at different pH in the presence of various detergents and organic solvents. We found that both acidic and basic pHs are beneficial for Bcl-x_L dimerization. High concentrations of non-ionic detergents and some organic solvents can significantly promote this event. In addition to non-covalently linked acidic-dimer as that formed at acidic pH, Bcl-x_L formed disulphide-bonded detergent-dimer at neutral and basic pH when incubated with high concentrations of non-ionic detergents. The acidic-dimer retains the BH3 peptide binding activity, whereas the detergent-dimer does not. The formation of acidic-dimer and detergent-dimer implies that Bcl-x_L may dimerize via two different pathways under certain conditions. The implications of these findings has been discussed with previous experimental results, which provides some new insight into the events and would help the experiment design and data interpretation when non-ionic detergents are used to study the dimerization and pore formation of Bcl-2 family proteins.

Key words: apoptosis, Bcl-2 family protein, dimerization, domain swapping, non-ionic detergent.

Abbreviations: BH2/3, Bcl-2 homology 2/3; DTNB, dithiobisnitrobenzoic acid; IPTG, isopropyl β-D-thiogalactoside; DPC, dodecylphosphocholine; PAGE, polyacrylamide gel electrophoresis.

Apoptosis is the programmed cell death to maintain the normal functioning of multicellular organisms. As the key regulator of apoptosis, Bcl-2 family protein controls the cell death by forming homo- or heterodimers among the anti-apoptotic and pro-apoptotic members of this family (*1*, *2*). For example, Bcl-x_L is an anti-apoptotic Bcl-2 family protein that is primarily localized on mitochondrial outer membranes (*3*). As it forms cation-selective channels in lipid vesicles, Bcl-x_L is suggested to maintain cell survival through ion-channel formation in mitochondrial membranes (*4*). Similarly, Bax is another important pro-apoptotic Bcl-2 family protein. At the onset of apoptotic stimuli, it undergoes a conformational change with the aid of a BH3-domain only protein, tBid. The allosteric Bax is translocated from cytosol into mitochondrial membranes, forming ion-channels or permeability transition pores (PTP) to release apoptotic factors out of mitochondria. It is suggested that Bcl-x_L may exert the anti-apoptotic activity by heterodimerizing with Bax to prevent the ion-channels or PTP formation (*5*).

Thus far, two potential models have been proposed for the dimerization of Bcl-2 family proteins. Peptides derived from BH3 domain of pro-apoptotic proteins could bind with anti-apoptotic proteins such as Bcl-2 or Bcl-x_L (*6*, *7*). The complex structures of BH3 peptides with Bcl-x_L have been solved by either NMR techniques or crystallography (*8–11*), all of which show that the peptides bind into the hydrophobic cleft constituted by BH3, BH1 and BH2 domain residues of Bcl-x_L. Therefore, it is proposed that Bcl-2 family proteins may form heterodimers by residing BH3 domain of pro-apoptotic proteins into the hydrophobic cleft of anti-apoptotic proteins. Meanwhile, the crystal structure of a truncated Bcl-x_L homodimer without C-terminal transmembrane region revealed that Bcl-x_L monomers can form into homodimers by reciprocally exchanging BH2 and C-terminal domains (*12*). However, cell experiments demonstrated that Bcl-x_L C-terminal transmembrane region also participated in the homodimerization and was required for the heterodimerization with Bax (*13*), adding greatly to the complexity of potential models to explain the dimerization of Bcl-2 family proteins.

To gain further information on these events, the present work is focused on Bcl-x_L homodimerization at different pH in the presence of various detergents and organic solvents. We found that high concentrations

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of non-ionic detergents and some organic solvents can significantly promote Bcl-x_L homodimerization. It forms non-covalently linked dimer at acidic pH and disulphide-bonded dimer at neutral and basic pH when incubated with high concentrations of non-ionic detergents. The implications of these findings has been discussed with previous experimental results, which provides some new insight into the events and would help the experiment design and data interpretation when non-ionic detergents are used to study the dimerization and pore formation of Bcl-2 family proteins.

MATERIALS AND METHODS

Mutagenesis—The expression plasmid for Bcl-x_L (C151A) mutant protein was derived from a pET32b vector coding for a truncated human Bcl-x_L without the C-terminal 22 residues. The forward and reverse primers for the mutation were 5′-GGGGTAAACTGGGCTCGCA TTGTGGCC-3′ and 5′-GGCCACAATGCGAGCCCAGTTT ACCCC-3′, respectively. The mutagenesis was carried out using QuickChange site-directed mutagenesis kit (Catalogue no. 200519, Stratagene Ltd., CA). The PCR reaction was performed with 16 cycles of amplification including the denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 6.25 min. Residual template plasmid was removed by digestion at 37°C for 1 h with 1 μl (10 U/μl) methylation-dependent endonuclease, Dpn1. The constructed plasmid for Bcl-x_L(C151A) mutant protein was transformed into *Escherichia coli* XL1-Blue supercompetent cells. Individual colonies that grew on LB-agar plates supplemented with 100 μg/ml ampicillin were inoculated into 1 ml LB culture medium, which was incubated at 37°C with shaking overnight. The expression plasmid for Bcl-x_L(C151A) mutant protein was confirmed by DNA sequence analysis.

Recombinant Protein Expression and Purification—The expression plasmid for C-terminal His-tagged Bcl-x_L or Bcl-x_L(C151A) mutant protein was transformed into *E. coli* BL21 competent cells. Single colony of bacteria was transferred into LB culture medium and grown to an A_{600} of 0.6. Protein expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 3 h at 37°C. Cell pellets were resuspended with lysis buffer (50 mM Tris, 200 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted by pulse sonication. After centrifugation at 15,000 rpm for 30 min, the supernatant was loaded onto a Ni^{2+}-nitrilotriacetate (Ni-NTA)-agarose column. After washing with 40 mM imidazole (20 mM Tris, 200 mM NaCl, pH 8.0), the protein was eluted with 500 mM imidazole (20 mM Tris, 200 mM NaCl, pH 8.0) and further purified by Superdex 75 gel filtration. The purity of the protein was determined ≥99% by silver stained polyacrylamide gel electrophoresis (PAGE).

Incubation of Recombinant Proteins and Electrophoresis Analysis—To prepare protein samples with a pH gradient, 1 mg/ml Bcl-x_L or Bcl-x_L(C151A) was dialysed in a series of buffers for 9 h at 4°C. Specifically, the dialysis buffers were 50 mM acetate (200 mM NaCl, pH 3.0–5.0), 50 mM MES (200 mM NaCl, pH 6.0), 50 mM HEPES (200 mM NaCl, pH 7.0), 50 mM Tris (200 mM NaCl, pH 8.0) and 50 mM Bicine (200 mM NaCl, pH 9.0 or 10.0). The samples were incubated either alone or with various additives at particular temperatures. The electrophoresis analysis was carried out on 10% polyacrylamide gel and visualized by silver staining method. The concentration of dithiothreitol (DTT) in reductive sample buffer was 200 mM. The pH of stacking gel and separating gel were 6.8 and 8.8, respectively. The concentration and volume of protein samples loaded into the wells of a gel were controlled to be the same.

Circular Dichroism Spectroscopy—To determine the melting temperature (T_m) of Bcl-x_L, the molar ellipticity of 10 μg/ml Bcl-x_L in distilled water was recorded at 222 nm at an average of four times scan in the temperature range of 30–90°C with the step of 0.5°C. The experiment was performed in a quartz cuvette with a 0.5 cm path-length on a JASCO 715 spectropolarimeter (JASCO Corp., Japan).

Raman Spectroscopy—Raman spectra in the range of 500–1800 cm^{-1} were recorded with an integration time of 60 s on a confocal micro-Raman spectrometer (T64000, Jobin-Yvon, France). The spectrometer was equipped with a 50 mW argon–krypton laser excitation source of 514 nm, a microscope (IX 81, Olympus, Japan), a holographic notch filter to reject Rayleigh scattering and a liquid nitrogen cooled CCD detector (CCD-3000V, Edison N.J., USA). A ×60 microscope water objective was used to focus laser and collect Raman scattering on the cells. The spectrometer was calibrated with the silicon phonon mode at 520 cm^{-1}. Raman spectra of Bcl-x_L (20 mM Tris, 200 mM NaCl, pH 8.0) with or without 1% Triton X-100 were subtracted of the contribution from solvent and normalized to the same protein concentration (10 mg/ml).

Detection of the Exposed Bcl-x_L Cysteine—1 mg/ml Bcl-x_L was prepared in 20 mM Tris buffer (pH 8.0) with or without 1% Triton X-100. After incubation at 37°C for 1 h, 100 μl sample solution was mixed with 10 μl 20 mM dithiobisnitrobenzoic acid (DTNB). The absorbance at 412 nm was recorded immediately by the time-course scanning mode on a U2010 spectrophotometer (Hitachi Ltd., Japan).

Chemical Cross-linking—1 mg/ml Bcl-x_L in 20 mM Tris buffer (200 mM NaCl, pH 8.0) was incubated with 1% Triton X-100 and CuP (0.4 mM $CuSO_4$, 1.6 mM phenanthroline) for various periods of time as indicated in figure. The reaction was stopped by mixing with SDS–PAGE non-reductive sample buffer that contains 20 mM *N*-Ethylmaleimide (NEM) and 20 mM EDTA. The reaction product was analysed on 10% SDS–PAGE in the absence of any reducing agent.

Binding Activity of Bcl-x_L Dimers with Bak BH3 Peptide—1 mg/ml Bcl-x_L in 20 mM Tris buffer (200 mM NaCl, pH 8.0) or in 50 mM acetate buffer (200 mM NaCl, pH 4.0) was incubated with 1% Triton X-100 at 37°C for 1 h. The samples were then mixed with 0.1 mM Bak BH3 derivative peptide with the sequence of SQVGRQL AIIGDDICR (C-Strong Co., Ltd., China) and analysed by native gel electrophoresis.

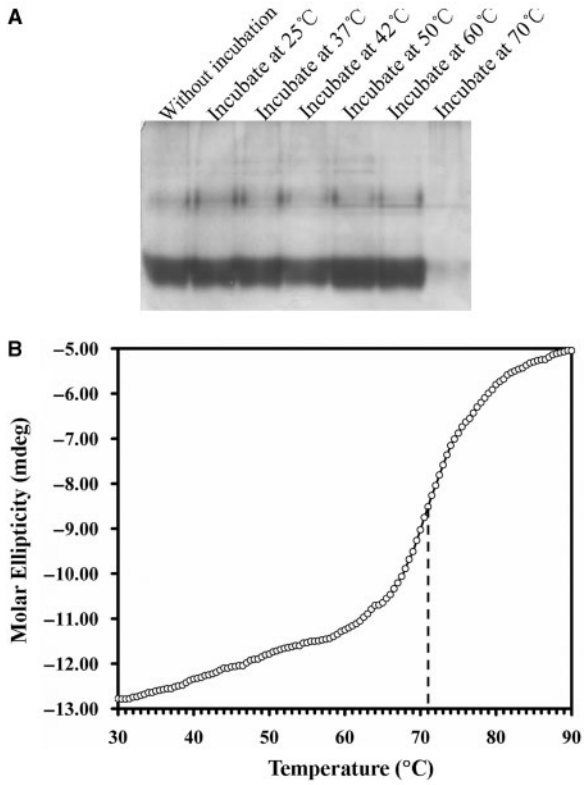

Fig. 1. **(A) Native gel analysis of Bcl-x_L (1 mg/ml, pH 8.0) incubated for 1 h at 25, 37, 42, 50, 60 and 70°C, respectively.** (B) The melting temperature of Bcl-x_L (10 μg/ml, distilled water) measured by CD spectroscopy at 222 nm.

RESULTS

Non-ionic Detergents and Some Organic Solvents Promote Bcl-X_l Homodimerization—As Bcl-x_L dimerization is important for its anti-apoptotic activity, we tried to promote this event by incubation of Bcl-x_L at various conditions so that it could be studied *in vitro*. First, we incubated Bcl-x_L monomer (pH 8.0) at different temperatures and identified the dimer formation by native gel analysis. Little amount of Bcl-x_L dimer was detected after 1 h incubation at 25, 37, 42, 50 and 60°C (Fig. 1A). The protein began to denature at 70°C, which is near its melting temperature (T_m) as shown by CD spectrum of Bcl-x_L (Fig. 1B). The incubation experiment suggested that simply raising the temperature did not significantly promote Bcl-x_L dimerization. We further incubated Bcl-x_L with various ions, detergents, denaturants and organic solvents. High concentration of NaCl (up to 1 M), 100 mM $CaCl_2$ or moderate concentration of bivalent metal ions such as 1 mM $CuSO_4$, 1 mM $NiSO_4$ failed to induce Bcl-x_L dimerization. Neither could denaturants such as 2 M urea or 1 M guanidine hydrochloride induce the dimerization. In contrast, incubation of Bcl-x_L with 1% Triton X-100 (Fig. 2A, lane 3), 20% (v/v) Tween-20 (Fig. 2B, lane 2), 30% (v/v) ethanol (Fig. 2C, lane 4) or 10% acetone (v/v) (Fig. 2D, lane 2) could induce the dimerization. Interestingly, even though the concentration of 0.1% Triton X-100 (*i.e.* 1.55 mM) is over its critical micelles concentration (CMC) (*i.e.* 0.24 mM), it did not induce Bcl-x_L dimerization (Fig. 2A, lane 2). To examine the kinetics of Bcl-x_L homodimerization, we incubated Bcl-x_L with 1% Triton X-100 for 1–4 h. We found that the amount of Bcl-x_L dimer increased in the first 2 h. As the incubation time was prolonged after that, the amount of Bcl-x_L dimer kept almost unchanged (Fig. 2E). We also incubated Bcl-x_L with 1% Triton X-100 at a series of temperatures for 1 h and found that Bcl-x_L formed dimer and oligomers at all these conditions (Fig. 2F). However, the amounts of Bcl-x_L monomer, dimer and multimers began to decrease at 60°C, suggesting that the treatment with 1% Triton X-100 had promoted Bcl-x_L unfolding and its precipitation at a temperature below T_m.

Bcl-x_L Dimers Formed at Acidic and Basic pH Showed Different Patterns on PAGE—There have been different reports regarding the pH effect on the dimerization of Bcl-2 family proteins. Some believed that acidic pH increased the binding affinity between Bcl-x_L monomers (*14*) while the others demonstrated that basic pH was beneficial for Bcl-x_L dimerization (*12*). To study the pH effect on the event, Bcl-x_L was dialysed against a series of buffers with pH in the range of 4.0–10.0. After incubation for 1 h at 37°C, we did see various amounts of Bcl-x_L dimer at these pH conditions. However, Bcl-x_L formed more dimers at acidic and basic pH (Fig. 3A). When Bcl-x_L was incubated with 1% Triton X-100 at 37°C, it displayed distinct degree of dimerization at these pH (Fig. 3B). Bcl-x_L at acidic pH ($pH \leq 5$) and basic pH ($pH \geq 8$) dimerized significantly in the presence of Triton X-100. In contrast, it formed little dimers at pH 6.0. These suggest that both statements about the pH effect on Bcl-x_L dimerization are correct. The difference is that Bcl-x_L more readily formed into dimer, trimer and/or higher molecular weight oligomers at $pH \geq 7$ compared to the dimeric and weak trimeric bands at acidic pH. Besides, Bcl-x_L dimer formed at $pH \geq 7$ in the presence of 1% Triton X-100 showed a slower dispersed band on native gel than the dimer formed at acidic pH. For brevity, Bcl-x_L dimers formed at $pH \geq 7$ in the presence of non-ionic detergents such as Triton X-100 and at acidic pH are termed detergent-dimer and acidic-dimer, respectively. It should be noted that not all Bcl-x_L dimers formed at $pH \geq 7$ were detergent-dimer. A minority of acidic-dimer was also observed at neutral pH (Fig. 3B).

Non-ionic Detergents Induced Disulphide-bonded Dimer Formation—To find out the reason why acidic-dimer and detergent-dimer displayed different patterns on native gel, Bcl-x_L incubated with 1% Triton X-100 at pH 4.0 and 8.0 were further analysed on SDS–PAGE. Since conventional SDS–PAGE disrupts non-covalent bond interactions of protein, only one band corresponding to Bcl-x_L monomer would be identified on SDS–PAGE if Bcl-x_L dimers were non-covalently connected. Interestingly, when DTT was omitted from the sample buffer, Bcl-x_L dimer bands were also detected on SDS–PAGE for Bcl-x_L incubated at pH 8.0 (Fig. 4A). A possible explanation is that the monomeric units in Bcl-x_L dimer are linked by a disulphide-bond. As the disulphide-bond is resistant to the treatment of SDS and boiling, detergent-dimer could survive on SDS–PAGE. On the other hand,

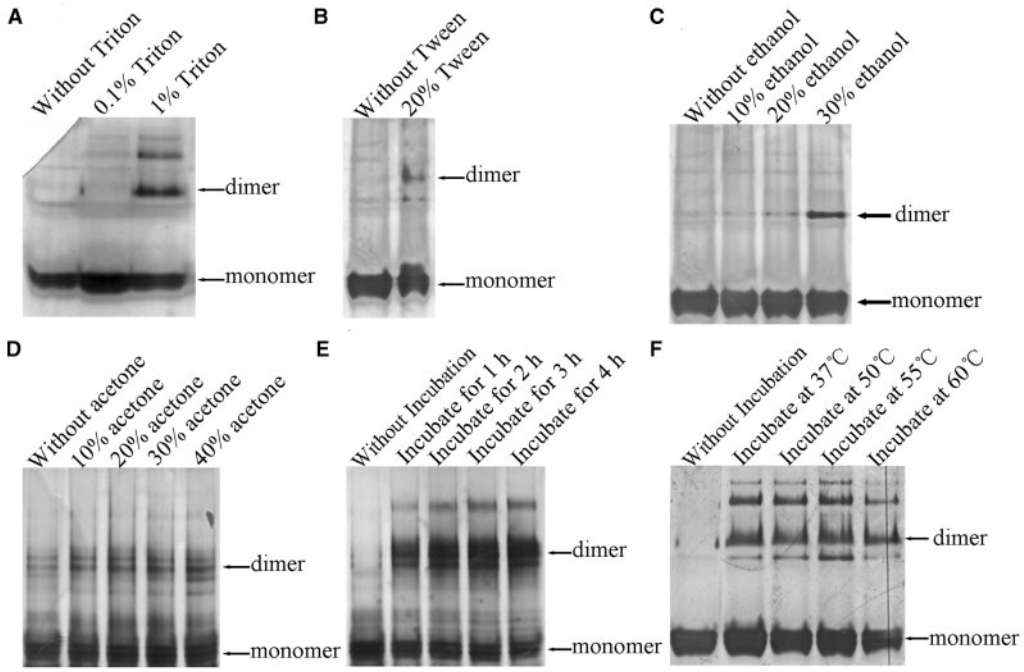

Fig. 2. **Native gel analysis of Bcl-x_L (1 mg/ml, 20 mM Tris, 200 mM NaCl, pH 8.0) incubated with non-ionic detergents or organic solvents.** (A) Bcl-x_L incubated at 37°C with Triton X-100 for 1 h; (B) Bcl-x_L incubated at 37°C with 20% Tween 20 for 1 h; (C) Bcl-x_L incubated at 37°C with ethanol for 1 h; (D) Bcl-x_L incubated at 37°C with acetone for 1 h; (E) Bcl-x_L incubated with 1% Triton X-100 for 1–4 h; (F) Bcl-x_L incubated with 1% Triton X-100 for 1 h at 37, 50, 55 and 60°C, respectively.

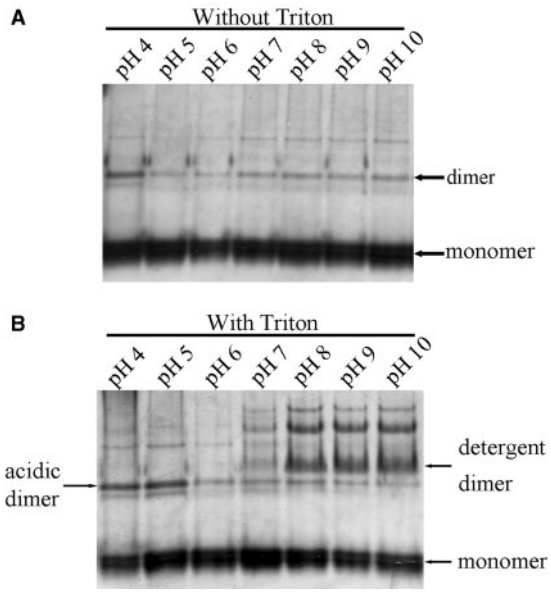

Fig. 3. **Native gel analysis of Bcl-x_L (1 mg/ml) incubated at 37°C for 1 h without Triton X-100 (A) or with 1% Triton X-100 (B) at different pH conditions.**

since DTT can break the disulphide-bond, Bcl-x_L dimer formed at pH 8.0 could not be detected on SDS–PAGE when it was loaded with reductive sample buffer (Fig. 4A), suggesting that the disulphide-bond links the monomeric units of Bcl-x_L detergent-dimer. At pH 4.0, the treatment with Triton X-100 did not give rise to the band of Bcl-x_L dimer on SDS–PAGE even when DTT and mercaptoethanol were omitted from the sample buffer (Fig. 4A), which could be explained by the non-covalent bond interactions between monomeric units of Bcl-x_L acidic-dimer. To further confirm this result, Bcl-x_L incubated with 1% Triton X-100 at pH 8.0 and 4.0 were analysed on native gel. When 1% mercaptoethanol was incubated with Bcl-x_L or 200 mM DTT was added into the sample buffer, Bcl-x_L detergent-dimer also disappeared on native gel (Fig. 4B), suggesting that reductive reagents such as DTT and mercaptoethanol had broken the disulphide-bond and therefore disassembled the detergent-dimer into monomers. As reductive reagents do not interfere with non-covalent bond interactions, acidic-dimer was still observed in the presence of either DTT or mercaptoethanol (Fig. 4C). We also examined Bcl-x_L dimers induced by 20% Tween 20. Likewise, the treatment with mercaptoethanol or DTT could eliminate the detergent-dimer formed at 20% Tween 20 (Fig. 4D).

Cysteine on α5 Helix is Involved in Detergent-dimer Formation—Recombinant Bcl-x_L protein has only one cysteine on α5 helix that is buried inside and usually not accessible for disulphide-bond formation unless a dramatic conformational change has caused the exposure of this residue. To confirm the involvement of the cysteine in detergent-dimer formation, we constructed a Bcl-x_L(C151A) mutant protein whose cysteine was substituted by alanine. The mutant protein was dialysed in a series of buffers with pH ranging from 3.0 to 10.0. After 1 h incubation at 37°C with 1% Triton X-100, we examined the dimer formation of Bcl-x_L(C151A) mutant protein by native gel analysis. Only one type

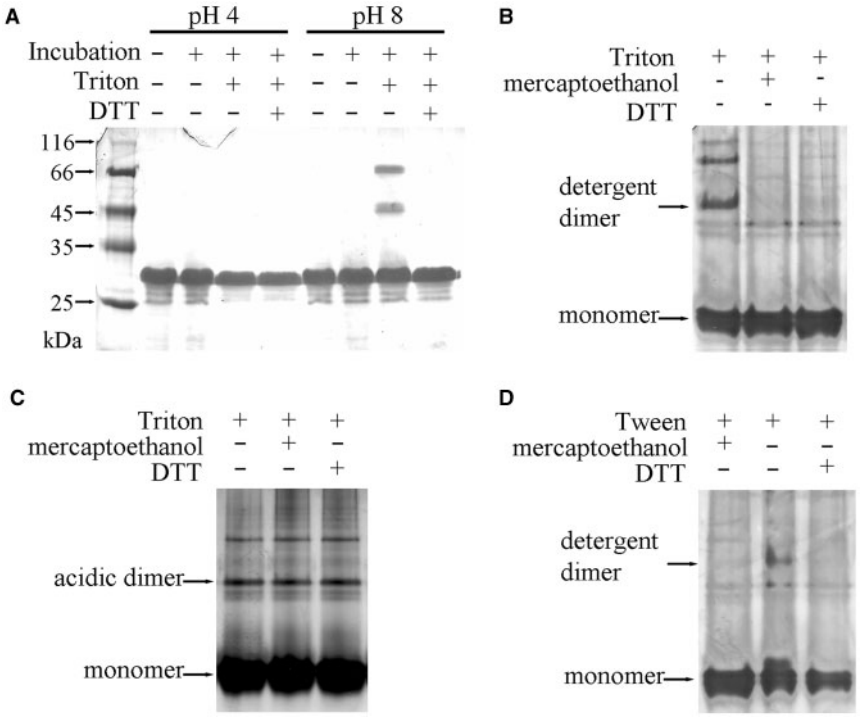

Fig. 4. **(A) SDS–PAGE analysis of 1 mg/ml Bcl-x_L at pH 4.0 and 8.0 incubated at 37°C with 1% Triton X-100 for 1 h.** (B) Native gel analysis of Bcl-x_L (1 mg/ml, pH 8.0) incubated at 37°C for 1 h with 1% Triton X-100 or with 1% Triton X-100 plus 1% (v/v) mercaptoethanol. (C) Native gel analysis of Bcl-x_L (1 mg/ml, pH 4.0) incubated at 37°C for 1 h with 1% Triton X-100 or with 1% Triton X-100 plus 1% (v/v) mercaptoethanol. (D) Native gel analysis of Bcl-x_L (1 mg/ml, pH 8.0) incubated at 37°C for 1 h with 20% Tween 20 or with 20% Tween 20 plus 1% (v/v) mercaptoethanol. The protein samples in the polyacrylamide gel electrophoresis were loaded with either non-reductive sample buffer or reductive sample buffer that contains 200 mM DTT.

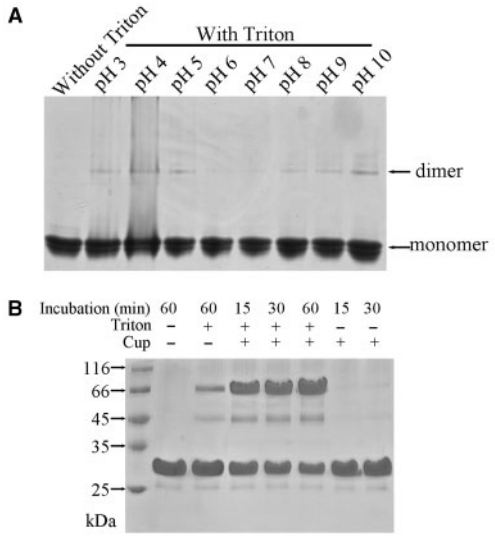

Fig. 5. **(A) Native gel analysis of Bcl-x_L(C151A) mutant protein (1 mg/ml, pH 3.0–10.0) incubated with 1% Triton X-100 at 37°C for 1 h.** (B) SDS–PAGE analysis of Bcl-x_L (1 mg/ml, pH 8.0) incubated with 1% Triton X-100 and CuP (0.4 mM $CuSO_4$, 1.6 mM phenanthroline) for different period of time.

of dimer was formed regardless of the pH value (Fig. 5A). The shift and pattern of the dimeric bands were the same as those for Bcl-x_L acidic-dimer. This could be explained by the fact that Bcl-x_L(C151A) mutant protein does not have cysteine. Therefore, it could not form disulphide-bonded dimer even at basic pH condition.

The involvement of the cysteine residue in detergent-dimer formation was also confirmed by chemical cross-linking experiment. When the cysteine residues of two Bcl-x_L monomers are in close positions, the sulphydryl groups can be oxidized into a disulphide-bond by the oxidant copper phenanthroline (CuP). Bcl-x_L incubated without non-ionic detergent and CuP, as the negative control, did not form SDS-resistant dimer (Fig. 5B, lane 2). In contrast, it formed SDS-resistant detergent-dimer in the presence of 1% Triton X-100 (Fig. 5B, lane 3). Here it should be mentioned that the molecular weight for the recombinant Bcl-x_L protein is 25 kDa. Similar to a previous report (*15*), a major Bcl-x_L monomer band near 30 kDa and a minor Bcl-x_L monomer band near 25 kDa were observed on SDS–PAGE, suggesting that the recombinant Bcl-x_L protein expressed from bacteria may adopt two different isoforms. Compared to the positive control (Fig. 5B, lane 3), the incubation with CuP in the presence of 1% Triton X-100 prompted Bcl-x_L to form more detergent-dimer that displayed a darker band near 66 kDa on SDS–PAGE. As the incubation time was prolonged from 15 min to 1 h, the monomeric band became thinner while the dimeric band near 66 kDa became stronger (Fig. 5B, lanes 4–6). In addition to the 66 kDa dimer band, we also observed a 45 kDa dimer band on SDS–PAGE (Figs 4A and 5B). The intensity of this 45 kDa-band increased a little when Bcl-x_L was

incubated with CuP (Fig. 5B, lanes 3 and 4), however it seems unchanged as the incubation time was extended to 1 h probably because the concentration of CuP was high enough to oxidize all the Bcl-x_L cysteine residues in this isoform within 15 min. Reducing reagents such as DTT did disrupt the 45 kDa dimer (Fig. 4A), suggesting that this dimeric form also requires the formation of disulphide-bond. To examine whether Bcl-x_L detergent-dimer was formed through the domain-swapping pathway (*12*), we incubated Bcl-x_L monomer with CuP in the absence of non-ionic detergent. Since heat induces Bcl-x_L to form domain-swapping dimer at basic pH (*16*), the treatment with CuP would oxidize the detergent-dimer formation if it came from the intermediate structures on the domain-swapping pathway. However, we did not observe any detergent-dimer at this condition (Fig. 5B, lanes 7 and 8), suggesting that Bcl-x_L detergent-dimer is not formed through the domain swapping.

Conformational Change of Bcl-x_L Induced by Triton X-100—At pH 8.0, free cysteine residue can react with DTNB to yield a yellow product that has a strong absorption at 412 nm (*17*). Using the Ellman reaction, we confirmed the exposure of Bcl-x_L Cys by the treatment of Triton X-100. To do so, Bcl-x_L (pH 8.0) was incubated with or without 1% Triton X-100 at 37°C for 1 h, respectively. Immediately after the addition of DTNB, the absorbance at 412 nm was measured at a fixed time interval for 30 min. The absorbance had little increase along with the time if Bcl-x_L was incubated without Triton X-100. However, it increased significantly if Bcl-x_L was incubated with Triton X-100 (Fig. 6A). An explanation for the phenomena is that the cysteine on α5 helix of Bcl-x_L is well buried inside and not accessible for the reaction with DTNB. After the incubation with Triton X-100, the cysteine became exposed and active for the reaction. This suggests that non-ionic detergents such as Triton X-100 had induced a conformational change of Bcl-x_L and caused the exposure of the buried cysteine.

The conformation properties of Bcl-x_L at pH 8.0 with or without 1% Triton X-100 were also characterized by Raman spectroscopy (Fig. 6B). The contributions from secondary structures of α-helix, β-sheet and random coil to the amide I band on Raman spectra occur near 1650–1655, 1665–1680 and 1655–1665 cm^{-1}, respectively (*18*). Previous NMR and X-ray studies demonstrated that monomeric Bcl-x_L mainly adopt the α-helical conformation (*15*). Accordingly, a strong and broad amide I band centred at 1656 cm^{-1} was observed on Bcl-x_L Raman spectra, showing that the secondary structure of Bcl-x_L is dominated by α-helix in the absence and presence of Triton X-100. In another word, the treatment with 1% Triton X-100 did not bring about a dramatic change upon the secondary structure of Bcl-x_L. The detergent-dimer induced by 1% Triton X-100 should be formed through the helix repacking of Bcl-x_L. Meanwhile, Raman spectra also reflect the environment change of Bcl-x_L residues. hBcl-x_L has seven tryptophans, among which Trp169 and Trp188 are buried inside, Trp137 and Trp181 are partially solvent accessible and the others are exposed to the solvent. The environment change of the tryptophan residues can be characterized by the intensity ratio of Raman bands at 1360 and 1340 cm^{-1}

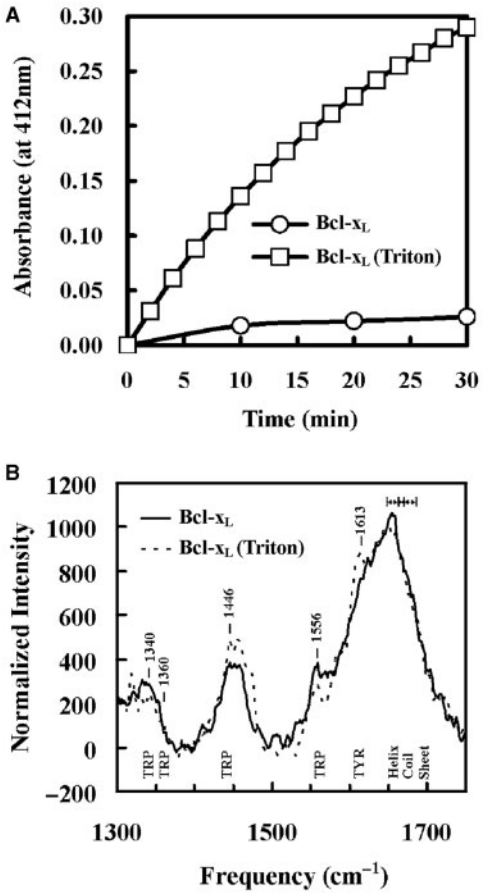

Fig. 6. **(A) Bcl-x_L (1 mg/ml, 20 mM Tris, 200 mM NaCl, pH 8.0) was incubated with or without 1% Triton X-100 at 37°C for 1 h.** The reaction of Cys-151 with DTNB was recorded by the absorbance at 412 nm in time course; (B) Raman spectra of Bcl-x_L with or without 1% Triton X-100 were normalized to the same protein concentration.

(*i.e.* I_{1360}/I_{1340}) (*19*). The ratio of I_{1360}/I_{1340} for Bcl-x_L in Tris buffer (pH 8.0) without Triton X-100 was 0.23. It became 0.52 when Bcl-x_L was prepared in 1% Triton X-100, meaning that the tryptophan residues were buried into a more hydrophobic environment.

BH3 Peptide Binding Activity of Acidic-dimer and Detergent-dimer—The BH3 domain of pro-apoptotic proteins is important for the heterodimerization with anti-apoptotic proteins. It has been shown that peptides derived from BH3 domain of pro-apoptotic proteins such as Bak, Bad and Bim bind into the hydrophobic cleft of Bcl-x_L (*8*, *9*, *11*). Bcl-x_L domain-swapping dimer retains the hydrophobic cleft and the binding activity with BH3 domain peptides (*12*). To examine the binding activity of Bcl-x_L acidic-dimer and detergent-dimer with BH3 peptide, monomeric Bcl-x_L was incubated in 1% Triton X-100 with or without CuP at pH 4.0 or 8.0, respectively (Fig. 7). The protein samples were mixed with a peptide derived from BH3 domain of Bak and then analysed on native gel. It is shown that monomeric Bcl-x_L could bind with the BH3 peptide at both acidic and basic pH regardless of the treatment of Triton X-100. Bcl-x_L acidic-dimer formed at pH 4.0 was also able to bind with

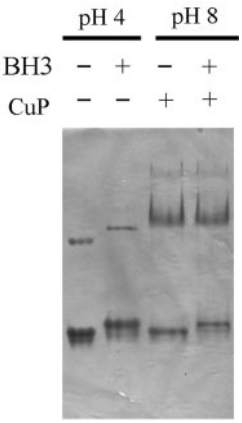

Fig. 7. **A total of 1 mg/ml Bcl-x_L (pH 4.0 or pH 8.0) was incubated with 1% Triton X-100 and CuP (0.4 mM $CuSO_4$, 1.6 mM phenanthroline) at 37°C for 1 h.** The samples were mixed with 0.1 mM Bak BH3 derivative peptide and analysed on 10% Tris–Glycine native gel.

the BH3 peptide, suggesting that the hydrophobic cleft remains intact and the acidic-dimer is formed probably through the domain-swapping pathway. In contrast, Bcl-x_L detergent-dimer formed at pH 8.0 in the presence of Triton X-100 did not bind the BH3 peptide, indicating the disappearance of the conserved hydrophobic cleft. Therefore, Bcl-x_L detergent-dimer was formed through a different pathway other than the domain-swapping.

DISCUSSION

Effect of pH, Detergents, and Organic Solvents on Bcl-x_L Homodimerization—The dimerization of Bcl-2 family proteins plays an important role in their regulation of apoptosis. To study the conformational change and dimerization of Bcl-x_L, we incubated Bcl-x_L monomer at a series of temperatures and with various denaturants, detergents and organic solvents. We found that simply raising the temperature had little effect on Bcl-x_L homodimerization. Low concentration of non-ionic detergents such as Triton X-100 even above its CMC did not induce the dimerization. In contrast, treatment with high concentrations of non-ionic detergents such as Triton X-100, Tween 20 or some organic solvents such as ethanol, acetone could significantly promote this event. These suggest that the native structure of Bcl-x_L is well packed. The structural transformation from monomer to dimer needs to go through a conformational change that features a high energy barrier. Once the conformational change was induced by non-ionic detergents and organic solvents, Bcl-x_L can readily form into homodimers even at physiologic temperature.

The conformational change of Bcl-x_L induced by non-ionic detergents could be demonstrated by the exposure of the conserved cysteine residue on α5 helix. As shown by the tertiary structures of Bcl-x_L (PDB ID: 1MAZ, 1LXL), the cysteine residue is buried inside and has a low reactivity with DTNB. After incubation with 1% Triton X-100 at 37°C for 1 h, the cysteine was exposed out and available for the reaction, suggesting that a major conformational change had happened with the structural region(s) around the cysteine. Comparing the solution structures of Bcl-2 and Bcl-x_L, the conserved Cys on α5 helix of Bcl-2 monomer is also buried inside and cannot be modified by thio-reacting probes as that of Bcl-x_L in the absence of non-ionic detergents. However, the Cys on α5 helix in membrane-anchored Bcl-2 was accessible for IASD labelling (*20*), suggesting that the conformational change with Bcl-2 had exposed the Cys out before its insertion into the membrane.

Apart from non-ionic detergents and some organic solvents, we found that both acidic and basic pH are also beneficial for Bcl-x_L dimerization, which is consistent with the previous report regarding the promotional effect of acidic pH on Bcl-x_L dimerization (*14*) and the NMR study demonstrating that the speed of the heat-induced Bcl-x_L homodimerization was 10^3 times faster at pH 10.0 than pH 7.0 at 30°C (*16*). Meanwhile, we also found that Bcl-x_L formed non-covalently linked dimer at acidic pH, whereas disulphide-bonded dimmer, at neutral and basic pH when incubated with high concentrations of non-ionic detergents. There is only one cysteine in Bcl-x_L that is located on α5 helix. The disulphide-bond formation indicated that α5 helix is at the contact interface between the monomeric units of Bcl-x_L detergent-dimer.

To exclude the possibility of His-tag-mediated association, we incubated the recombinant Bcl-x_L protein with metal ions such as Cu^{2+} and Ni^{2+}. Neither metal ion could induce Bcl-x_L dimerization. We also incubated the recombinant protein with chelating agent EDTA to get rid of any trace amount of metal ion. Incubation with 1 mM EDTA did not affect Bcl-x_L dimerization (Supplementary data). Therefore, the dimerization of Bcl-x_L protein is not mediated by the C-terminal His-tag. The effect of pH, detergents and organic solvents on Bcl-x_L dimerization is the intrinsic property of the protein.

Bcl-x_L Can Form Homodimers via Two Distinct Pathways—The secondary structure and mobility of Bcl-x_L in zwitterionic detergent dodecylphosphocholine (DPC) (pH 7.0, PBS buffer) has been characterized by NMR techniques (*21*). Though the ionic detergent did not induce Bcl-x_L homodimerization, the hydrophobic interior of DPC micelle bound with α1, α6 and probably partial α5 helices of Bcl-x_L, causing a dramatic conformational change of the protein. Consequently, the cysteine residue on α5 helix was transformed into a flexible turn structure. As shown by our DTNB experiment, the cysteine of Bcl-x_L was exposed out by the treatment with 1% Triton X-100. One explanation for the exposure is that α5 helix of Bcl-x_L was collapsed into a flexible turn structure as in the case of zwitterionic detergent. Another explanation is that non-ionic detergents had caused the intermediate structures formation of Bcl-x_L on the domain-swapping pathway. As the result, α6 helix was dislocated from the core structure so that the cysteines of two Bcl-x_L monomers were accessible for the disulphide-bond formation. The third possible explanation is that a major conformational change with Bcl-x_L had exposed α5 and α6 helices out, which is similar to the event of their insertion into intracellular membranes. Therefore, α5 helices of two Bcl-x_L monomers could get close at the half side with the cysteine.

As the treatment with zwitterionic detergent did not induce Bcl-x_L homodimerization, the collapse of partial α5 helix into a flexible structure is not sufficient for detergent-dimer formation. There must be some other structural rearrangements induced by non-ionic detergents. Raman spectra indicated that the treatment with 1% Triton X-100 did not bring about a dramatic change upon the secondary structure of Bcl-x_L. The detergent-dimer might be formed through the helix repacking. The second explanation may also be excluded in considering the chemical cross-linking experiment results. Since Bcl-x_L at basic pH forms homodimers through domain-swapping as revealed by NMR study (*16*), the incubation of Bcl-x_L with CuP at basic pH in the absence of Triton X-100 should result in disulphide-bond dimer if it came from the intermediate structures on the domain-swapping pathway. However, no SDS-resistant dimer was observed on SDS–PAGE at this condition. Meanwhile, the detergent-dimer of Bcl-x_L lost the BH3 peptide binding activity, clearly indicating that the dimerization did not go through the domain-swapping pathway. The third explanation does not conflict with the present data, but further experiments such as spectroscopy study on Bcl-x_L protein in non-ionic detergents and membranes are needed to verify this explanation.

Unlike the detergent-dimer, Bcl-x_L acidic-dimer retains the BH3 peptide binding hydrophobic cleft, implying that it may be formed through the domain-swapping pathway. It is believed that Bcl-x_L may form multimers by domain swapping through the head-to-tail connections. Our *in vitro* incubation experiments showed that Bcl-x_L formed acidic-dimers and few trimers at acidic pH in the presence of non-ionic detergent (Fig. 3B). However, it readily formed into detergent-dimers, trimers and/or higher order oligomers at neutral and basic pH, suggesting that Bcl-x_L at this condition is more prone to oligomerization. We also observed that Bcl-x_L trimer and/or higher order oligomers formed at this condition were susceptible to reductive reagents though Bcl-x_L has only one cysteine for the disulphide-bond formation (Fig. 4B). This may be understood by suggesting that the detergent-dimer is the 'building block' for Bcl-x_L trimer and/or higher order oligomers. The disulphide-bond in detergent-dimer is critical for maintaining the right structural features to stabilize non-covalent interactions between detergent-dimers or 'detergent-dimer and monomer' in multimers. Moreover, as shown on Raman spectra, high concentrations of non-ionic detergents such as 1% Triton X-100 created a hydrophobic environment surrounding the tryptophan residues of Bcl-x_L, which likely mimics the insertion into intracellular membranes (*22*). The high oligomerization propensity of Bcl-x_L at neutral pH in 1% Triton X-100 might help understand its behaviour after the insertion into mitochondrial membranes. Here, it should be noticed that Bcl-x_L formed only one type of homodimer at acidic and basic pH in the absence of Triton X-100 (Fig. 3A). But it formed non-covalently linked dimer at acidic pH, whereas disulphide-bonded dimmer, at neutral and basic pH when incubated with high concentrations of non-ionic detergents. This means that the treatment with non-ionic detergents had differentiated the dimerization pathway of Bcl-x_L.

Implications in the Dimerization of Bcl-2 Family Proteins—Non-ionic detergents such as Triton X-100 and β-octylglucoside (OG) could convert Bax monomer into oligomers and induce its dimerization with other Bcl-2 family proteins (*23*, *24*). However, neither Bcl-2 nor Bcl-x_L monomers could oligomerize in detergents including Triton X-100 (*23*, *25*). In contrary to the previous reports, here we have demonstrated that high concentrations of non-ionic detergents could as well promote Bcl-x_L homodimerization and oligomerization.

In addition to the promotional effect, non-ionic detergents had also been reported to inhibit the interactions of Bcl-x_L mutant proteins with Bax. Specifically, non-ionic detergent 0.2% NP-40 did not affect the binding activity of Bax with Bcl-x_L by GST-Bax pull-down assay. However, it greatly reduced the binding affinity of Bax with Bcl-x_L(F131V) and completely abolished the binding activity with Bcl-x_L(V135A/N136I/W137L) mutant protein (*26*), though none of the mutated residues is involved in the interaction with BH3 peptide according to the complex structures of Bcl-x_L. This discrepancy may be resolved by the destabilized folding structures of Bcl-x_L mutant proteins, which become more susceptible to forming into detergent-dimer that would lose the BH3 peptide binding activity. In contrast, wild-type Bcl-x_L is more resistant to the effect of non-ionic detergents and did not form into detergent-dimer at low concentrations of non-ionic detergents such as 0.1% Triton X-100 or 0.2% NP-40. This is supported by the fact that Bcl-x_L(F131V) in 0.2% Triton X-100 appeared at 66 kDa on size-exclusion chromatography, which is similar to the 66 kDa detergent-dimer of wild-type Bcl-x_L in 1% Triton X-100 (Figs 4A and 5B). When HA-Bcl-x_L or HA-Bcl-x_L(F131V) was absorbed on protein A-agarose beads, co-immunoprecipation experiment showed that they had similar Bax binding affinity at non-ionic detergents, indicating that the immobilization had made Bcl-x_L(F131V) more resistant to the conformational change induced by non-ionic detergents (*26*).

It seems perplexing that Bcl-x_L detergent-dimer formed in 1% Triton X-100 did not bind the BH3 peptide while non-ionic detergents could promote the Bcl-x_L/Bax interaction. Actually, this can be explained by considering the concentration of the non-ionic detergent. Low concentration of Triton X-100 (e.g. 0.1%) did not promote the detergent-dimer formation. Even at high concentration of Triton X-100, majority of Bcl-x_L existed as monomers *in vitro*. As shown in Fig. 7, Bcl-x_L monomer treated with 1% Triton X-100 still possessed the BH3 peptide binding activity at acidic and basic pH. The promotional effect of non-ionic detergent on Bcl-x_L/Bax interaction should come from the conformational change of Bax that facilitates the binding of Bax BH3 domain residues into the hydrophobic cleft of Bcl-x_L (*24*).

Besides, it has been reported that oligomeric Bax but not monomeric Bax was able to form channels and trigger the release of cytochrome c from liposomes or

isolated mitochondria (*23*, *27*). The oligomeric Bax was either purified from bacteria with 1% Triton X-100 at pH 8.0 or prepared by the incubation of monomeric Bax in 2% OG or 1% Triton X-100. The recombinant protein was finally dialysed in buffer containing 0.2 mM DTT. Typically, 1–10 mM DTT was used to keep cysteine residues in reduced state during protein purification. A total of 0.2 mM DTT may not efficiently prohibit the disulphide-bond formation. As the evidence, Bax oligomerization through this process appears to be irreversible. Bax oligomer 160 kDa was dissociated into monomer and dimer on SDS–PAGE (*23*). As discussed before, 1% Triton X-100 induced Bcl-x_L to form SDS-resistant detergent-dimer. Likely, Bax protein induced by high concentrations of non-ionic detergents may also form into detergent-dimer and multimers that facilitate the channel formation in liposomes. In contrast, CHAPS did not induce the oligomerization of Bax. Monomeric Bax treated with CHAPS failed to form channels in liposomes. Further study on Bax dimerization induced by high concentrations of non-ionic detergents as conducted in our present work would provide more details about the event and help elucidate Bax channel formation in membranes.

In conclusion, combining the approaches of PAGE, chemical cross-linking, Raman spectroscopy and Ellman reaction, we have studied the conformational change and homodimerization of Bcl-x_L protein. Both acidic and basic pHs are found beneficial for Bcl-x_L homodimerizaton. High concentrations of non-ionic detergents and some organic solvents can significantly promote this event. In addition to non-covalently linked dimer as that formed at acidic pH, Bcl-x_L formed SDS-resistant disulphide-bonded dimer at neutral and basic pH when incubated with high concentrations of non-ionic detergents. The acidic-dimer retains the BH3 peptide binding activity, whereas the detergent-dimer does not. The formation of acidic-dimer and detergent-dimer implies that Bcl-x_L may form homodimers via the domain-swapping pathway as revealed by Bcl-x_L homodimer crystal structure and another distinct pathway that needs to be characterized with further studies. Meanwhile, as the cysteine on α5 helix of Bcl-x_L is conserved among many Bcl-2 family proteins, it is possible that other Bcl-2 family members may form into detergent-dimers as Bcl-x_L. Therefore, the present work would help the experiment design and data interpretation when non-ionic detergents are used to study the dimerization and pore-formation of Bcl-2 family proteins.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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