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α-Lactalbumin Forms with Oleic Acid a High Molecular Weight Complex Displaying Cytotoxic Activity[†]

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ABSTRACT: α-Lactalbumin (LA) forms with oleic acid (OA) a complex which has been reported to induce the selective death of tumor cells. However, the mechanism by which this complex kills a wide range of tumor cell lines is as yet largely unknown. The difficulty in rationalizing the cytotoxic effects of the LA/OA complex can be due to the fact that the molecular aspects of the interaction between the protein and the fatty acid are still poorly understood, in particular regarding the oligomeric state of the protein and the actual molar ratio of OA over protein in the complex. Here, the effect of LA addition to an OA aqueous solution has been examined by dynamic light scattering measurements and transmission electron microscopy. Upon protein addition, the aggregation state of the rather insoluble OA is dramatically changed, and more water-soluble and smaller aggregates of the fatty acid are formed. A mixture of LA and an excess of OA forms a high molecular weight complex that can be isolated by size-exclusion chromatography and that displays cellular toxicity toward Jurkat cells. On the basis of gel filtration data, cross-linking experiments with glutaraldehyde, and OA titration, we evaluated that the isolated LA/OA complex is given by 4−5 protein molecules that bind 68−85 OA molecules. The protein in the complex adopts a molten globule-like conformation, and it interacts with the fatty acid mostly through its α-helical domain, as indicated by circular dichroism measurements and limited proteolysis experiments. Overall, we interpret our and previous data as indicating that the cellular toxicity of a LA/OA complex is due to the effect of a protein moiety in significantly enhancing the water solubility of the cytotoxic OA and, therefore, that the protein/OA complex can serve mainly as a carrier of the toxic fatty acid in a physiological milieu.

α-Lactalbumin (LA)[1] is an acidic calcium binding protein (14.2 kDa, 123 residues) involved in the biosynthesis of lactose in the mammary gland, being a component of the lactose synthase complex (*1*). The three-dimensional structure of the calcium-bound form of LA is characterized by two domains, a discontinuous helical domain given by chain segments 1−34 and 86−123 encompassing the four main α-helices (denoted A−D) of the protein and a sheet/coil *β*-domain given by the rest of the protein chain (*2*). Since few decades LA has been extensively used as a model system in protein folding studies (*3*, *4*), as well as for highlighting the molecular features of the interaction of proteins with liposomes, micelles, and membranes (*5*−*10*). In particular, the molten globule (MG) state that LA acquires at acidic pH or upon removal of calcium at neutral pH at low salt concentration and moderately high temperature has been extensively analyzed by means of a variety of techniques and approaches (*1*, *3*, *4*, *11*−*15*), thus being considered nowadays a paradigm of partly folded states of proteins (*3*, *11*). An interesting property of this protein is its ability to interact with oleic acid (OA), forming a LA/OA complex that can induce apoptosis in tumor cells but not in healthy cells (*16*−*23*). The mechanism of this toxicity is still not clarified, since several cellular targets appear to be involved in the observed biological activity (*24*−*28*) (see also Rammer et al. (*29*) for additional references). Nevertheless, the use of the LA/OA complex for cancer therapy appears to be quite promising, considering the positive results obtained in treating glioblastomas (*30*), skin papillomas (*31*), and bladder cancer (*32*, *33*).

The biologically active LA/OA complex was initially isolated from human milk and shown to be given by a multimeric form of LA and, therefore, named MAL (multimeric alpha-lactalbumin) (*16*−*20*). Interestingly, the protein moiety of MAL displayed the conformational features of a MG-like state, in much analogy to that adopted by LA at low pH (*3*, *11*). Subsequently, it was shown that human LA can be converted to the biologically active form also *in vitro*, upon loading the protein in the apo form on an anion-exchange chromatographic column conditioned with OA and eluting the LA/OA complex by a 1−1.5 M NaCl concentration, followed by extensive dialysis and lyophilization (*21*). This *in vitro* prepared active complex was named HAMLET (human alpha-lactalbumin made lethal to tumor cells). An active complex, named BAMLET, can be prepared also with the bovine

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[1]Abbreviations: LA, α-lactalbumin; BLA, bovine LA; OA, oleic acid; HAMLET and BAMLET, human or bovine α-lactalbumin made lethal to tumor cells; apo-BLA, Ca^{2+}-depleted bovine LA; CD, circular dichroism; MAL, multimeric LA; MG, molten globule; CMC, critical micelle concentration; EDTA, ethylenediaminetetraacetic acid; E/S, enzyme to substrate ratio; ESI-MS, electrospray ionization−mass spectrometry; HPLC, high-performance liquid chromatography; RP, reverse phase; RT, retention time; [*θ*], mean residue ellipticity; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; FTIR, Fourier-transform infrared spectroscopy; DLS, dynamic light scattering.

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protein (BLA), as well as with equine, porcine, ovine, and caprine LA (*34*). These LA/OA complexes were all quite similar to MAL in terms of conformational and biological properties, but at variance from the multimeric protein observed in the active species isolated from milk (MAL), in HAMLET the protein was reported to be mainly monomeric and to bind one molecule of fatty acid (*23*, *35*−*37*). Also, a model of the 1:1 molar interaction between LA and OA was proposed on the basis of NMR, H/D exchange, and limited proteolysis experiments (*35*−*37*). However, it is worth noting that the MG-like conformation that LA acquires upon binding OA appears to be unusual for a fatty acid binding protein (*38*), being instead more similar to that observed when this protein interacts with liposomes or micelles (*5*−*10*).

In all studies originating from the Svanborg's laboratory the active OA complex was prepared by the chromatographic procedure and not by simple mixing of the two components. However, we (*39*, *40*) and others (*41*, *42*) have reported that the mixing procedure can also produce OA complexes that display similar conformational and biological properties to those of HAMLET-like samples prepared by the chromatographic procedure. Depending upon the method used for preparing the complex, it was reported that human LA can bind "irreversibly" 2.6 or 9 OA molecules if the complex formation is achieved by mixing the two components at 17 or 45 °C, respectively (*42*), whereas a 1/5.1−5.4 LA/OA molar ratio was recently evaluated in the HAMLET complex prepared by chromatography (*43*). Moreover, in a recent paper Rammer et al. (*29*) reported a very detailed study of the biological properties of BAMLET prepared by the chromatographic procedure, still assuming that the protein/fatty acid ratio in the complex was 1:1. Overall, it is clear that a coherent view of the molecular properties of the complex is still lacking. No doubt that a fruitful comparative analysis of the results so far obtained in Svanborg's (*24*, *26*−*28*) and other laboratories (*29*, *39*, *44*−*48*) will be possible only once the molecular details of the complex will be clarified.

Here, we have prepared the complex by direct mixing of BLA and OA in solution, and the active BLA/OA complex was isolated by gel filtration chromatography. The BLA/OA complex is shown to be composed by a protein oligomer interacting with a micellar-like aggregate of the fatty acid and that in the complex the protein adopts a MG-like conformation, as deduced from circular dichroism (CD) measurements. We show here that the protein addition to an OA solution significantly enhances the water solubility of the fatty acid. Considering that OA is poorly soluble in aqueous solution at neutral pH (*40*, *49*−*53*) and has intrinsic cytotoxic properties (*54*, *55*) (see also Figure 6 in Knyazeva et al. (*42*)), we propose that the interaction of the protein with OA leads to an enhanced solubility of the fatty acid in a physiological milieu, thus making the LA/OA complex a suitable delivery system of the toxic OA to the cell. Since the protein moiety in HAMLET-like complexes appears to act as a carrier for OA, it can be proposed that great variability in the protein chain can be tolerated, in agreement with the fact that several LAs displaying only 63% sequence identity (*34*), mutants of LA (*43*, *56*) and proteolytic fragments of BLA (*40*), as well as equine lysozyme (*57*, *58*), can produce HAMLET-like complexes with similar biological properties.[2]

MATERIALS AND METHODS

Preparation of the BLA/OA Complex. A suitable amount of OA (Fluka) was dispersed in phosphate buffer, pH 7.4, upon vortexing, and the pH was adjusted to 8.0 with a solution of 0.1 M NaOH. The solution was stirred overnight at room temperature and then extruded through a 50 nm polycarbonate membrane filter just before mixing with the protein solution. Typically, the stock solution of OA at pH 8.0 was 60 mM. To prepare a concentrated protein/OA mixture for gel filtration chromatography experiments, bovine LA (BLA, type I, calcium saturated; Sigma) was dissolved in phosphate buffer, pH 7.4, containing 20 mM EDTA, and then mixed with an aliquot of OA stock solution in order to reach the desired BLA/OA molar ratio. The final pH was eventually adjusted to 7.4. The concentration of OA in the mixture was determined by FTIR (see below) and that of the protein by measuring the absorbance at 280 nm. The composition of phosphate buffer, pH 7.4, was 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 0.137 M NaCl. The BLA/OA mixtures were incubated for 1 h at room temperature before use.

Gel Filtration Chromatography. Analyses of BLA/OA complexes, prepared by mixing the protein and the fatty acid at different molar ratios, were performed on a Sephadex G-150 column (1.0 × 48 cm; Pharmacia) equilibrated and eluted with phosphate buffer, pH 7.4, at a flow rate of 4.8 mL/h. The absorbance of the effluent from the column was recorded at 226 nm. Samples were routinely loaded onto the column at a protein concentration of 15−25 mg/mL.

Dynamic Light Scattering and Turbidity Measurements. Dynamic light scattering (DLS) measurements were performed with a Malvern Zetasizer Nano-S (Malvern, Worcestershire, U.K.). Data were collected at 25 °C on an extruded solution of OA (65 mM) at pH 8.0 and on a BLA/OA mixture at a molar ratio of 1/15 (BLA 1.4 mM, OA 21 mM). OA concentrations are those calculated on the basis of the weighted fatty acid. Turbidity measurements were performed as previously reported (*40*).

Transmission Electron Microscopy. For negative staining transmission electron microscopy (TEM) analyses, a drop of the sample solution was placed on a Butvar-coated copper grid (400 square mesh) (TAAB Laboratories Equipment Ltd., Berks, U.K.), dried, and then negatively stained with a drop of uranyl acetate solution (1% w/v). TEM pictures were taken on a Tecnai G2 12 Twin instrument (FEI Co., Hillsboro, OR), operating at an excitation voltage of 80 kV.

FTIR Measurements. The concentration of OA was determined by FTIR spectroscopy as described by Walde et al. (*53*). Measurements were performed at room temperature using a Perkin-Elmer 1720X spectrometer purged with a continuous flow of N_2 gas. For each sample 25 interferograms were accumulated at a spectrum resolution of 2 cm^{-1} using a CaF_2 cell of 0.1 mm. In order to extract OA from the BLA/OA complex, 500 μL of sample solution in phosphate buffer, pH 7.4, was mixed with 500 μL of 1 N HCl and 400 μL of isooctane and then vortexed for 10 min. The resulting mixture was left at room temperature overnight and then centrifuged three times at 13400 rpm for 30 min in order to separate the OA-containing isooctane upper phase from the aqueous phase. The concentration of OA was determined in the organic phase on the basis of the intensity of the

[2]This work was presented at the XXI Symposium of the Protein Society (July 21−25, 2007, Boston, MA), *Protein Sci.16* (Suppl. 1), Communication 453, and at the Symposium on HAMLET (May 12−14, 2009, Lund, Sweden).

IR band at 1712.4 cm^{-1} in the FTIR spectrum of the isooctane solution, using an extinction coefficient of 855 M^{-1} cm^{-1} (*53*). The accuracy of measurements was determined using OA solutions of known concentration.

Chemical Cross-Linking. Samples of the BLA/OA complex or BLA alone at a protein concentration of 0.1 mg/mL in phosphate buffer, pH 7.4, were mixed with a freshly prepared 2.5% solution of the cross-linking agent glutaraldehyde (Fluka) in phosphate buffer to a final 0.01% concentration of the reagent. The reaction was allowed to proceed at room temperature, and aliquots were taken at intervals from the reaction mixture, stopped by addition of 1 M Tris buffer, and lyophilized. The protein samples were then analyzed by the Tris−glycine SDS−PAGE system under reducing conditions (*59*). Oligomers of BLA were prepared by cross-linking with glutaraldehyde a mixture of BLA/OA at a molar ratio of 1/18. The reaction was conducted as described above, and it was quenched after 7 h reaction with 1 M Tris. The solution was then dialyzed against 0.5 mM EDTA and 0.5 M NH_4HCO_3, pH 8.0, and dried in a vacuum concentrator. The cross-linked oligomeric species were purified by gel filtration chromatography on a Superdex 75 gel filtration column (Pharmacia) equilibrated and eluted with phosphate buffer, pH 7.4, at a flow rate of 0.5 mL/min. The absorbance of the effluent from the column was recorded at 214 nm. The lyophilized reaction mixtures were loaded onto the column after dissolution in 6 M Gdn·HCl. The chromatographic fractions containing the oligomeric species were collected and concentrated using an Amicon ultracentrifugal filter device with a 10 kDa cutoff (Millipore).

Circular Dichroism. Circular dichroism (CD) measurements were performed at room temperature on a Jasco J-710 spectropolarimeter (Tokyo, Japan). Near-UV CD spectra were recorded at a protein concentration of about 0.5 mg/mL using a quartz cell with a path length of 1 cm, whereas for spectra in the far-UV region protein solutions at a concentration of 0.1 mg/mL and a cuvette with a 0.1 cm path length were used. The results were expressed as mean residue ellipticity [θ] (deg·cm^2·$dmol^{-1}$). Protein concentration was determined by measuring the absorbance at 280 nm and using the extinction coefficient of BLA calculated according to Gill and von Hippel (*60*).

Cytotoxicity Tests. Human lymphoma Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2−95% air at 37 °C. Jurkat cells (10^6 cells/mL) were incubated with 0.1 mg/mL of the various BLA/OA samples or BLA alone in serum-free medium for up to 17 h at 37 °C. OA was tested at the same concentration as that of the multimeric species. To avoid any precipitation of the fatty acid, it was added to the cells just after dilution of the stock solution at pH 8.0 in phosphate buffer, pH 7.4. In order to assess cell viability, Jurkat cells were stained with 10 μM Hoechst 33258 or 1 μM propidium iodide for 5 min. Cells were then washed with Hanks' balanced salt solution, pH 7.4, and visualized with an Olympus IMT-2 inverted microscope, including a xenon light source (75W) and a 12-bit digital cooled, charge-coupled device camera (Princeton Instruments, Monmouth Junction, NJ). Fluorescent cell images were obtained with 20× immersion objectives (Nikon) using excitation/emission cubes of 340/440 25 nm and 568/585 25 nm long-pass filter for Hoechst 33258 and propidium iodide, respectively. Three randomly selected fields were acquired from each treatment. The corresponding bright field images were also acquired, and the three channels were overlaid using the appropriate function of the Metamorph software (University Imaging, West Chester, PA). All data are expressed as the mean ± sem.

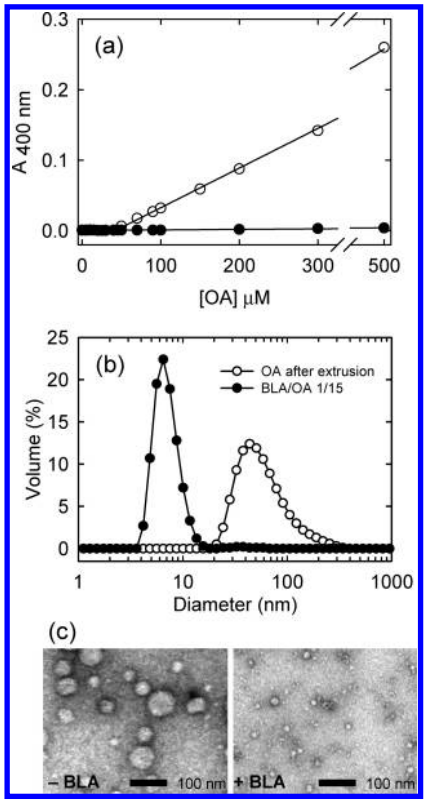

FIGURE 1: Characterization of the changes in the aggregation state of OA after addition of BLA. (a) Turbidimetric analysis of OA solutions in phosphate buffer, pH 7.4, in the absence (open circles) and in the presence of 10 μM protein (filled circles). The absorbance of the solutions was recorded at 400 nm on samples containing OA up to 500 μM. (b) Dynamic light scattering (DLS) size distribution of a solution of OA and of a mixture of BLA/OA at a molar ratio of 1/15. Data were obtained at room temperature on an extruded solution of OA (65 mM) at pH 8.0 and on a BLA/OA mixture at a molar ratio of 1/15 (BLA 1.4 mM, OA 21 mM) at pH 7.4. OA concentrations are those calculated on the basis of the weighted fatty acid. The size distributions of OA aggregates before and after addition of apo-BLA are indicated with empty and solid circles, respectively. (c) Negative staining TEM images of samples of OA in the absence (−BLA) and in the presence of BLA (+BLA).

RESULTS

BLA Forms a High Molecular Weight Species in the Presence of OA. Previous studies have shown that OA can form micelles at pH above 10.5 and that micelles spontaneously transform into vesicles if the pH is decreased below 9.0 (*49*−*53*). At pH lower than 8.0 the fatty acid forms large aggregates and in phosphate buffer, pH 7.4, a very low concentration of free OA is expected, considering that the critical micelle concentration (CMC) is ~20 μM (*40*, *49*, *50*). The aggregation of OA upon increasing its concentration in aqueous solution can be followed by turbidity measurements at 400 nm (*40*, *61*). As shown in Figure 1a, at variance from a solution of pure OA, in the presence of protein the fatty acid does not form the large aggregates that cause light scattering. The solubilizing effect for OA of the added protein is also simply shown by the fact that a turbid OA suspension in water at neutral pH becomes clear upon adding the protein. Since the molecular assemblies or vesicles of OA that form upon lowering the pH below pH 8.0 have a rather broad distribution of sizes and

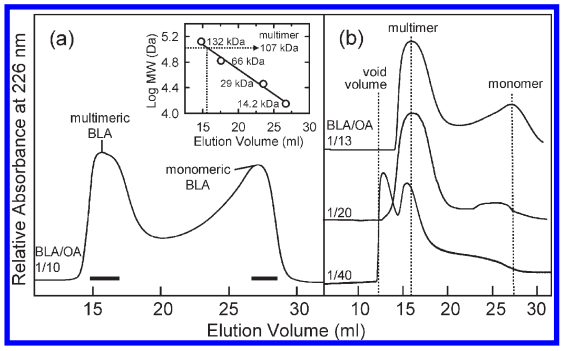

FIGURE 2: Gel filtration chromatography of the BLA/OA complex. (a) Gel filtration chromatographic analysis of a sample of BLA/OA at a molar ratio of 1/10. Solid lines indicate the fractions of the effluent from the column containing multimeric and monomeric BLA which were collected for further experiments. The insert shows the calibration curve of the Sephadex G-150 column obtained using as protein standard bovine serum albumin (66 kDa) and its dimer (132 kDa), carbonic anhydrase (29 kDa), and BLA (14.2 kDa). A dotted line indicates the elution volume of the multimeric species and the corresponding molecular mass. (b) Chromatographic analyses of a mixture of OA and BLA at different ratios of protein and fatty acid. Dotted lines indicate the void volume of the column and the elution volumes of the multimeric and monomeric species. The analyses were performed on a Sephadex G-150 column equilibrated in phosphate buffer, pH 7.4.

can form large aggregates, solutions of OA were extruded through a 50 nm polycarbonate filter before use in order to use homogeneous solutions of the fatty acid (*53*). Dynamic light scattering (DLS) measurements revealed that an extruded OA solution contains molecular assemblies of the fatty acid with a hydrodynamic mean diameter of 65.14 ± 5 nm, while in a mixture of BLA/OA at 1/15 molar ratio these OA assemblies have a mean diameter of 7.13 ± 0.98 nm (see Figure 1b). Also transmission electron microscopy (TEM) revealed a significant reduction of the size of the OA aggregates in the presence of added protein (Figure 1c). Therefore, the protein exerts a profound effect on the aggregation behavior of OA, in analogy to similar effects observed when proteins such as tau protein (*62*) or α-synuclein (*63*) are added to solutions of fatty acids or anionic detergents.

Solutions of OA and apo-BLA were mixed at different molar ratios in order to prepare the rather concentrated protein samples (~20 mg/mL) that subsequently were to be analyzed by gel filtration chromatography. The apo form of the protein was used, since the holoprotein does not seem to produce active complexes (*21*, *42*). After 1 h incubation at room temperature, the protein/OA mixtures thus obtained were clear, indicating the formation of an OA vesicle population of smaller size. Analysis of a mixture of BLA/OA at a molar ratio of 1/10 on a Sephadex G-150 gel filtration column yielded the chromatogram shown in Figure 2a. The protein material elutes from the column in two major chromatographic peaks, one early eluted corresponding to an oligomeric state of the protein and the other later eluted corresponding to the protein monomer. Calibration of the gel filtration column with proteins of known molecular mass allowed us to evaluate a molecular mass of ~110 kDa for the oligomer (Figure 2a, insert). The yield of the multimeric protein species with respect to that of monomeric BLA can be increased by using BLA/OA mixtures of relatively higher molar ratios of OA over protein, as shown by gel filtration data (see Figure 2b). Moreover, at a 1/40 molar ratio of BLA over OA, an additional chromatographic peak eluting with the void volume of the gel filtration column is observed, indicating the presence of additional species of higher molecular mass. Nonetheless, in this study we focused only on the characterization of the ~110 kDa multimer, which is the main species formed under relatively low BLA/OA molar ratios.

The samples of the BLA/OA complex used here are those early eluted from the Sephadex G-150 column upon loading a mixture of BLA/OA at a molar ratio of 1/10−1/20 containing 15−25 mg/mL protein (see Figure 2). Gel filtration chromatography of a 10-fold diluted sample of the BLA/OA mixture leads to a chromatogram where only the monomeric protein elutes from the column, suggesting that the oligomeric state of the BLA/OA complex is a quite dynamic aggregate in rapid exchange with the monomeric protein and that during gel filtration the oligomeric species is disrupted (Supporting Information Figure S1). This observation explains also the asymmetry and broadness of the gel filtration peak of the multimer (Figure 2). On the other hand, analysis by DLS of a 10-fold diluted BLA/OA mixture gave a mean diameter of OA assemblies identical to that of a nondiluted sample (see above), thus indicating that a 10-fold dilution does not affect the distribution of the macromolecular assemblies in solution.

Characterization of the Isolated BLA/OA Complex. Gel filtration chromatography of a 1/10 molar ratio mixture of BLA over OA can be used to isolate both multimeric and monomeric protein species, thus allowing further characterization (see Figure 2a). The molecular features of the high molecular weight species of BLA eluted from gel filtration (Figure 2a, first eluting protein material) were analyzed in terms of BLA/OA molar ratio and oligomeric state of the protein. The BLA/OA molar ratio was determined on the basis of actual protein content by measuring the protein absorbance at 280 nm and the OA concentration by FTIR (Supporting Information Figure S2) (*53*). The experimental error of the FTIR analytical procedure was ~5% for an OA solution, while ~15% in the presence of protein. From these measurements it was evaluated that in the isolated BLA/OA complex each protein molecule binds 17 ± 0.6 OA molecules, as determined from four independent analyses.

Chemical cross-linking experiments with glutaraldehyde were used to probe the oligomeric state of BLA in the presence of OA (*64*). To this aim, the time courses of the glutaraldehyde reactions performed on both the multimeric and monomeric species prepared by gel filtration (see Figure 2a) were analyzed by SDS−PAGE (Figure 3). The multimeric species suffers progressive cross-linking during the reaction, and the monomer disappears from the solution after 1 h reaction. The main protein bands seen in the gel after 420 min reaction indicate the formation of covalently bound protein oligomers given by three to six molecules of BLA, the more populated protein species being tetramers and pentamers. Conversely, SDS−PAGE analysis of the glutaraldehyde-mediated reaction conducted on the monomeric BLA sample isolated by gel filtration showed that protein cross-linking does not occur, since only a faint band of about ~28 kDa is seen in the gel even after 420 min reaction. This band corresponds to a protein dimer, which can form in the presence of the cross-linking agent from random collisions of protein monomers. A mixture of BLA and OA at 1:10 molar ratio and the apo form of BLA (not gel filtered) displayed a pattern of cross-linking similar to the multimeric and monomeric species obtained after gel filtration, respectively (data not shown), thus indicating that the BLA/OA complex is formed in solution upon simple mixing of the two components.

We have evaluated that the BLA/OA complex appears to be mainly composed of 4−5 protein molecules that associate with

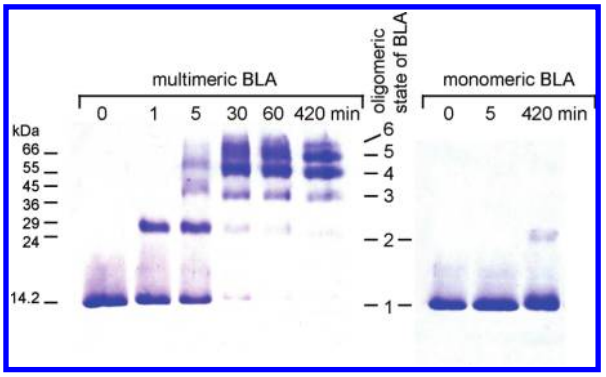

FIGURE 3: Cross-linking of BLA and its OA complex with glutaraldehyde. Multimeric and monomeric BLA collected after gel filtration chromatography (see Figure 2a) were cross-linked with glutaraldehyde at a reagent's 0.01% concentration and quenched at different times by adding a solution of 1 M Tris buffer. Cross-linked products were separated by SDS−PAGE and visualized using Coomassie blue stain. On the left side of the gels the molecular masses of the protein standards are indicated, whereas the number of cross-linked BLA molecules is shown between the two gels.

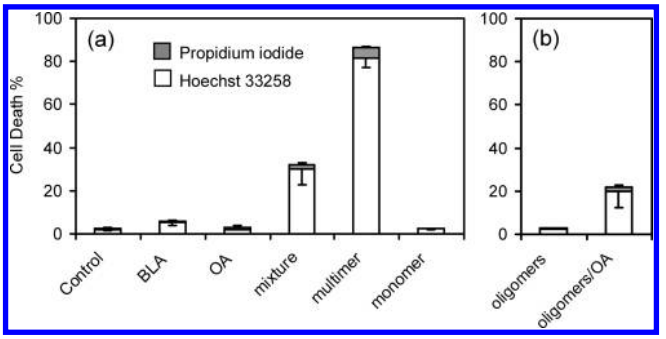

FIGURE 4: Cytotoxicity of the BLA/OA complex on Jurkat cells. Cells were incubated with BLA, a mixture of BLA/OA (molar ratio 1/10), and multimeric and monomeric BLA separated by gel filtration (a), as well as cross-linked BLA oligomers and a mixture of BLA oligomer/OA (molar ratio 1/10) (b). For all of these samples, the protein was tested at a concentration of 0.1 mg/mL, and cell death was measured after 17 h incubation using Hoechst 33258 (open bars) to stain apoptotic cells and propidium iodide (solid bars) to visualize cellular death due to necrosis. As a control, OA was tested at a concentration 113 μM, which corresponds to the concentration of the fatty acid in the multimeric fraction of the BLA/OA complex isolated after gel filtration. The percent of cell death was calculated from three acquisitions of each treatment. All data are expressed as the mean, with sem as error bars.

68−85 molecules of OA. The calculated masses are from 76 kDa for the tetramer to 95 kDa for the pentamer and up to 114 kDa for the hexamer, in essential agreement with the molecular mass of ~110 kDa calculated from gel filtration data (see above). These mass values of the protein assembly fit also with the mean diameter of the complex estimated by DLS, if one considers the expected volume of both LA and OA. Indeed, the volume of an OA molecule can be estimated to be ~447 Å^3, using Voronoi volume calculations from the X-ray structure of a fatty acid molecule bound in an extended conformation to rat liver fatty acid-binding protein (PDB code 1LFO) (*65*). The volume of a BLA molecule in the complex can be approximated to that of a sphere corresponding to the 2.08 nm Stokes radius of BLA in its MG state (*66*). Therefore, the volume occupied by a BLA/OA aggregate composed of 5 protein molecules and 85 fatty acid molecules can thus be calculated as ~226 nm^3, which is similar to the 203.6 ± 28 nm^3 volume of a sphere with the mean diameter of ~7.13 nm determined by DLS (see Figure 1b).

The Multimeric BLA/OA Complex Induces Apoptosis in Jurkat Cells. We next examined the effect of the multimeric and monomeric fractions of BLA/OA eluted from the Sephadex G-150 gel filtration column (Figure 2a) on cell viability of Jurkat cells, which were previously used for the biological testing of HAMLET (*16*, *18*, *21*). A comparative analysis was conducted also on samples given by the mixture of BLA and OA (not fractionated by gel filtration), apo-BLA, and OA. The cellular toxicity was assessed with Hoechst 33258 to visualize apoptotic cells and propidium iodide to stain the necrotic ones, and the results were expressed in terms of percent of cell death (Figure 4a). The multimeric BLA/OA species caused a dramatic loss of cell viability, while the monomeric species isolated from the gel filtration column negligibly affected cell viability. Also, native apo-BLA or OA alone did not appear to have a significant cytotoxic effect under the experimental conditions of the apoptosis test. The mixture of BLA and OA (not gel filtered) displays significant apoptotic activity but less than that of the purified multimeric BLA/OA complex, as expected from the fact that the sample mixture contains both active multimeric and inactive monomeric species and a lower OA concentration.

Cross-Linked Oligomers of BLA Display Cytotoxic Properties Only If Complexed with OA. The protein oligomers of the BLA/OA complex were stabilized by chemical cross-linking with glutaraldehyde (*64*) and purified by gel filtration chromatography (Supporting Information Figure S3). The gel filtration chromatogram of the cross-linked sample displays a main peak (fraction 1, see Supporting Information Figure S3a) mainly given by covalently linked tetramers and pentamers of the protein, as evidenced by SDS−PAGE analysis (Supporting Information Figure S3b). The fraction of the oligomeric species was collected and mixed with OA under the same experimental conditions used to prepare the mixture of BLA/OA (see above). Analysis by TEM of the oligomer/OA mixture evidenced a reduction in size of the aggregates of OA with respect to the solution of the fatty acid devoid of protein (Supporting Information Figure S4), in analogy to the similar effect observed with the monomeric protein (Figure 1c). As shown in Figure 4b, the cross-linked oligomers alone did not display cytotoxic properties toward Jurkat cells, but when mixed with OA they significantly affected cell viability. These results indicate that the covalently linked oligomers of BLA are not directly involved in the toxicity of the BLA/OA complex but that they require mixing with OA in order to display cytotoxicity.

BLA in the OA Complex Adopts a MG-like Conformation. The far- and near-UV CD spectra of the mixture of BLA/OA at a molar ratio of 1/10 and of the multimeric and monomeric BLA species collected after gel filtration chromatography (Figure 2a), as well as that of BLA at pH 2.0, are shown in Figure 5. Monomeric BLA isolated by gel filtration shows at room temperature far- and near-UV CD spectra that are superimposable to those of apo-BLA (not shown) and are characteristic of a native-like conformation of the protein. Indeed, it is well established that apo-BLA adopts a MG-like state only at low ionic strength and upon moderate heating (*1*, *39*). On the other hand, the BLA/OA mixture (not gel filtered) and multimeric BLA isolated after gel filtration show far-UV CD spectra characterized by an increase in the CD amplitude in the 203−215 nm region and a shape of the CD spectrum similar to that of the MG state of the protein at pH 2.0 (Figure 5a). In the near-UV region, both the multimer and the mixture show a

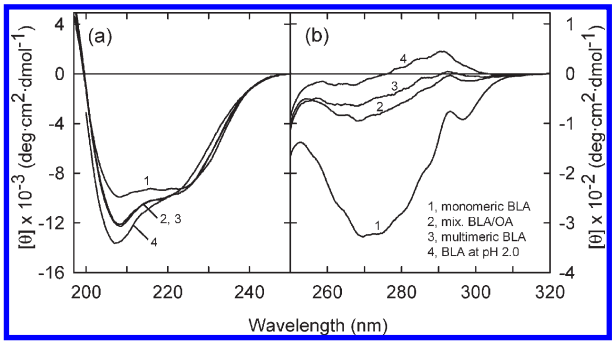

FIGURE 5: Spectroscopic characterization by circular dichroism of BLA and its complex with OA. Far- and near-UV CD spectra (a and b, respectively) of BLA at pH 2.0, of the mixture of BLA/OA (molar ratio 1/10), and of multimeric and monomeric BLA collected after gel filtration. Spectra were recorded at room temperature in phosphate buffer, pH 7.4, except for the spectrum of the mixture which contained also 0.3 mM EDTA and that of BLA recorded in 0.01 M HCl and 0.1 M NaCl, pH 2.0.

marked reduction of the negative ellipticity in the 260−300 nm region, resembling that of BLA at pH 2.0 (Figure 5b). The reduction of the CD signal is somewhat enhanced in the multimer with respect to that of the mixture, since likely the gel filtration step can eliminate the excess of monomeric protein from the mixture. Overall, these CD spectra indicate that in the isolated multimeric BLA/OA complex the protein adopts a MG-like conformation (*3*, *11*, *39*).

The effect of adding calcium on the protein conformation in the BLA/OA complex was investigated by CD measurements in the near-UV CD region (Supporting Information Figure S5). Interestingly, addition of an excess of $CaCl_2$ to the solution of the complex changes the MG-like conformation of the protein into a native one, as given by the resulting native-like near-UV CD spectrum. Moreover, we have observed that the addition of $CaCl_2$ to the solution of the BLA/OA complex develops some turbidity, likely due to the generation of the rather insoluble calcium oleate (*67*). Nonetheless, by adding an excess EDTA over calcium ions, the BLA/OA solution becomes transparent, and the resulting near-UV CD spectrum of the protein again resembles that of the active BLA/OA complex. These data indicate that the complex is a rather dynamic state that can be modulated by the presence of calcium ions and/or ion chelating agents.

Conformational Features of the BLA/OA Complex Probed by Limited Proteolysis. Limited proteolysis experiments have been applied previously to analyze the molecular features of partly folded states of BLA (*13*, *39*, *68*, *69*). Here, we have used this technique in order to determine in more detail the conformational changes induced on BLA upon addition of OA under the specific experimental conditions of this study. The proteolysis reactions were performed on the monomeric BLA and multimeric BLA/OA sample collected from gel filtration (see above and Figure 2a). We have also conducted proteolysis experiments on apo-BLA and on mixtures of BLA and OA (not gel filtered), and the results were essentially identical to those of protein samples after gel filtration (data not shown). Chymotrypsin and proteinase K were used as proteolytic probes, the first displaying a substrate specificity mostly for hydrophobic residues and the second instead a very broad substrate specificity (*69*). The details of the limited proteolysis experiments are given in the Supporting Information of this report and shown in Supporting Information Figures S6, S7, and S8.

Briefly, it was shown that the rate of proteolysis at room temperature of BLA in the complex is enhanced in respect to that observed with the apo form of BLA. The enhanced rate is explained by the fact that the protein in the complex adopts a dynamic and MG-like conformation (see Figure 5), while apo-BLA at 20−22 °C retains mostly a native-like conformation and only at temperatures >40 °C is the protein in a MG state (*1*, *39*). The initial proteolytic cuts of the 123-residue chain of BLA occur at the level of chain region 34−57, outside the α-helical segments of the native protein (see Supporting Information Figure S8). Finally, it was observed that the N- and C-terminal regions of the protein in the BLA/OA complex are rather resistant to proteolysis even after several hours of reaction. Overall, limited proteolysis experiments conducted on the BLA/OA complex were similar to those reported earlier for the MG state of BLA, since proteolysis of both protein samples occurs at the level of the coil/sheet region of the β-domain of native BLA (see Supporting Information Figure S8) (*13*, *39*). Thus, proteolytic probes indicate that this chain region is flexible or unfolded, in agreement with the structural features of the MG state of BLA previously deduced from other physicochemical measurements (*3*, *4*, *11*−*14*, *39*).

Comparison of the BLA/OA Mixture with a Sample of BAMLET. Here, we aimed also to compare the properties of a sample of BAMLET prepared by the OA-conditioned anion-exchange column chromatography according to Svensson et al. (*21*) with the BLA/OA samples herewith analyzed. The BAMLET species (Supporting Information Figure S9) resulted in a BLA/OA molar ratio of 1/8, as given by determining the protein and OA content in the sample (see above). Gel filtration of BAMLET (Supporting Information Figure S10) yielded a chromatogram similar to that obtained with the 1/10 molar ratio mixture of BLA/OA (see Figure 2), thus indicating that similarly BAMLET contains a multimer of ∼110 kDa molecular mass. BAMLET, as well as the multimeric and the monomeric species collected after gel filtration, was also characterized by near-UV CD spectroscopy (Supporting Information Figure S11) and toxicity tests (Supporting Information Figure S12). The results of these experiments demonstrated that the conformational and biological properties of BAMLET are similar to those exhibited by the BLA/OA complex prepared by mixing the two components.

DISCUSSION

The Active BLA/OA Complex Is Made by an Oligomeric Protein. Here, we demonstrate that BLA inhibits the formation of the large aggregates that OA spontaneously forms in phosphate buffer, pH 7.4, and that smaller and more soluble assemblies are formed in the presence of the protein. The protein-mediated enhanced solubility of OA is also simply observed by the fact that the added protein renders transparent a turbid solution of the fatty acid. Similar results were previously reported by studying the interaction between extruded vesicles of OA and lysozyme, a protein structurally related to BLA (*70*). It was found that lysozyme reduces the size of OA vesicles, forming a lysozyme/OA complex with a mean diameter of 10−20 nm, but the molecular features of this complex were not analyzed in detail. The BLA/OA complex can be formed by mixing the protein and fatty acid at 1/10−1/20 molar ratio, and it can be isolated by gel filtration chromatography (see Figure 2). On the basis of the results obtained by cross-linking experiments, quantitative analysis of protein over OA ratio, and dimensions

of the complex deduced from DLS measurements, we evaluate that the isolated BLA/OA complex herewith investigated is mainly composed by 4−5 molecules of protein and 68−85 molecules of OA.

While an oligomeric state of the protein was reported in the initial studies describing the isolation of the active LA/OA complex from human milk (*16*−*19*), subsequently gel filtration chromatography was used to demonstrate that the protein is monomeric in the active LA/OA complex prepared *in vitro* by the chromatographic method, named HAMLET (*21*, *35*−*37*). However, in this study we provide evidence that the LA/OA complex is a labile and dynamic protein/fatty acid complex that does not survive under the gel filtration chromatographic conditions, if a diluted sample is run onto the column (see Supporting Information Figure S1). Dissociation of labile protein complexes during chromatography is a well-known phenomenon, since only strongly associated molecules can elute from a gel filtration column as unique molecular species. Also, micelles and vesicles of OA are stable during gel filtration only if the fatty acid is loaded onto the column in relatively high concentrations (*53*). The rather concentrated protein/fatty acid mixtures analyzed in the present gel filtration experiments (15−25 mg/mL) favor the intermolecular associated state, so that the high molecular weight complex of BLA/OA can be eluted from the column. However, it should be emphasized that, even if gel filtration experiments of diluted BLA/OA samples reveal the presence of mostly protein monomers, the oligomeric nature of the protein is retained in BLA/OA samples also at a lower concentration. Indeed, a 10-fold dilution of the gel-filtered BLA/OA sample does not change the DLS-determined hydrodynamic mean diameter of the complex, and moreover, cross-linking experiments using glutaraldehyde, conducted at a 0.1 mg/mL protein concentration, clearly reveal the oligomeric nature of the protein in the active BLA/OA complex (see Figure 3).

We propose that previous studies failed to detect the presence of an oligomeric protein in HAMLET-like complexes due to the fact that too diluted samples were analyzed by gel filtration. This applies also to our previous gel filtration data for a BLA/OA sample prepared by mixing the two components in solution (*39*). Indeed, we show here that an active high molecular weight complex can be isolated by gel filtration of a BAMLET sample prepared by chromatography, provided that a relatively concentrated sample of the complex is analyzed (see Supporting Information Figure S10). Since gel filtration data indicate that the multimeric BLA/OA complex is in equilibrium with the monomeric protein (see Figure 2), the possibility that the monomer could bind OA can be considered, in view of the fact that the apo form of BLA, but not the holo one, can bind OA in a 1:1 molar ratio at neutral pH with an association constant for OA of $4.6 \times 10^6\ M^{-1}$ (*71*). At any rate, the monomeric protein isolated after gel filtration does not display cytotoxicity (see Figure 4), nor a 1:1 mixture of protein and fatty acid (*37*).

Here, we have shown that the mixing procedure can be used to prepare an active BLA/OA complex with conformational and biological properties similar to the complex produced using the anion-exchange chromatographic method of Svensson et al. (*21*). We may also mention that it has been recently demonstrated that even equine lysozyme can form an OA complex with cytotoxic properties similar to those of HAMLET- or BAMLET-like complexes and that the lysozyme/OA complex can be prepared by the chromatographic method (*57*, *58*). The lysozyme/OA complex was shown to be multimeric by NMR diffusion experiments, and in different preparations, the complex was shown to be given by 4−30 lysozyme molecules, each binding 11−48 molecules of OA (*57*). Even if the lysozyme/OA complex displays a great variability in composition, its molecular features appear analogous to those of the multimeric BLA/OA species herewith investigated.

The Protein Is Partly Folded in the BLA/OA Complex. The conformational features of the protein in the BLA/OA complex resemble those of the MG state of the protein previously investigated (*3*, *11*−*15*). The far- and near-UV CD spectra of the multimeric BLA/OA complex are very similar to the CD spectra obtained with BLA in its classical MG state at pH 2.0 (see Figure 5). In previous studies, using a variety of biophysical techniques and approaches, it has been amply demonstrated that the MG of BLA is characterized by a folded α-domain and a flexible or largely unfolded β-domain. In particular, we have previously shown that the structural features of the MG of BLA deduced from limited proteolysis experiments nicely agree with those reached by using other physicochemical and spectroscopic data (*13*, *39*, *68*, *69*). Limited proteolysis experiments reveal that in the BLA/OA complex the β-domain region is more rapidly attacked by proteases than the rest of the 123-residue chain of the protein (see Supporting Information Figure S8c), indicating that the chain region encompassing the β-domain is rather flexible (*68*, *69*). Conversely, the α-helical regions of the protein (helix A, helix D, and part of helices B and C) are quite resistant to proteolysis, suggesting that they are involved in the interaction with OA aggregates.

On the basis of the limited proteolysis data we can propose a mechanism of interaction between the protein and OA aggregates. The association between BLA and OA is likely to be mediated by both electrostatic and hydrophobic interactions. The apo form of BLA is negatively charged at physiological pH, and thus it is not expected to interact with the negatively charged surface of OA aggregates. Nonetheless, even such a protein molecule may possess positively charged clusters or areas that mediate the interaction with the negatively charged headgroups of OA aggregates. In BLA at neutral pH, Lys and Arg residues are clustered at the level of helical segments A, C, and D of the native protein (10 residues over a total of 12), and indeed these chain regions appear to preferentially interact with OA aggregates. There are only two positively charged residues (Lys58 and Lys79) in the chain segment 31−90, which instead contains up to 11 negatively charged carboxylates of Asp and Glu (see Supporting Information Figure S8c). It could be well that, in the presence of negatively charged OA aggregates, the region roughly from residues 31 to 90 remains loosely bound to the fatty acid due to electrostatic repulsion and thus more flexible than the rest of the 123-residue chain of the protein. Indeed, limited proteolysis occurs faster at the level of this chain region (see Supporting Information Figure S8).

The partly folded conformation of the protein in the BLA/OA complex resembles that reported for the membrane-bound state of the protein (*5*). Hydrogen−deuterium exchange measurements demonstrated that the protein bound to negatively charged phopholipid bilayers shows exchange kinetics similar to that of the MG state of BLA, but some residues located in helices A and C remain protected due to the interaction with the membrane (*6*, *7*).

BLA Oligomers Are Cytotoxic Only in Association with OA. A purified oligomer of 4−5 BLA molecules covalently linked by the glutaraldehyde reaction can display cytotoxic activity only if complexed with OA, thus emphasizing the role

of the fatty acid in the observed toxicity. Thus, even this randomly cross-linked, high molecular weight protein species can form an active OA complex. Of interest, in a very recent paper it has been reported that heating holo BLA at 80 °C for up to 100 min causes extensive protein denaturation and aggregation and that a high proportion of the protein aggregates were linked by disulfide bonds (*48*). Nevertheless, active complexes of thermally aggregated BLA were prepared using the chromatographic method of Svensson et al. (*21*), and their cytotoxic properties were very similar to those of a BAMLET sample prepared using native apo-BLA. Therefore, the results obtained here with glutaraldehyde-cross-linked oligomers parallel those obtained with a disulfide-cross-linked protein (*48*).

It has been reported that OA can induce the formation of toxic protein aggregates if the apo form of BLA is incubated at pH 4.0−4.5 in the presence of 640 μM OA at 37 °C (*44*). The protein aggregates thus formed were separated by centrifugation and analyzed for their toxicity on human lung tumor cells. Evidence of protein-aggregated forms (up to tetramers) was reached on the basis of mass spectrometric measurements. The authors placed emphasis on the toxicity of the LA oligomers as being related to the toxicity of the early protein aggregates formed by amyloidogenic proteins (*72*, *73*). However, it cannot be excluded that the aggregated protein material analyzed for toxicity by Zhang et al. (*44*) also contains protein-bound OA, thus making these aggregated LA samples analogous to the toxic protein/OA species herewith investigated.

Cytotoxicity of the BLA/OA Complex. The selective *in vitro* tumoricidal effect of HAMLET-like complexes toward different tumor cell lines has been reported in series of publications (*22*−*28*), but the molecular mechanisms of the cytotoxic effects are largely unclear. Actually, numerous unrelated mechanisms have been advanced, including apoptosis, interaction with proteasomes, mitochondrial membrane permeabilization, autophagic cell death, histone binding, and consequent DNA damage. Nevertheless, the therapeutic effect of HAMLET has been confirmed *in vivo* in a human glioblastoma xenograph rat model, as well as in human patients with skin papillomas and bladder cancer (*31*−*33*) (see Rammer et al. (*29*) for references).

In previous studies, the specific role of the protein moiety in the biological effects mediated by HAMLET-like complexes was emphasized (*24*, *25*, *43*). It was shown that the protein should be devoid of protein-bound calcium and in a MG-like conformation in order to be able to form the active OA complex. To this aim, mutants unable to bind calcium, as the Asp87Ala mutant (*56*), and even an all-Ala mutant with all eight Cys residues replaced by Ala (*43*) have been produced and studied for their capacity to form active OA complexes. The OA complexes of these LA variants of human LA were prepared using the chromatographic method (*21*), and all displayed biological effects quite similar to those observed with the wild-type protein. Moreover, all LA variants of human, bovine, equine, ovine, caprine, and porcine origin can be used for preparing OA complexes that display similar cytotoxic properties, despite the fact that these protein variants show only 71% homology and 63% identity of amino acid sequence (*34*). Here, we show that even a heavily glutaraldehyde-cross-linked multimeric species of BLA if associated with OA can display cytotoxicity, in analogy to the denatured and disulfide-cross-linked oligomer of BLA prepared by thermal treatment of the holoprotein (*48*). Finally, also several fragments of BLA prepared by limited proteolysis of the protein can produce cytotoxic OA complexes (*40*). All of these data clearly indicate that the protein moiety does not have a specific role in the biological properties of the complex but rather that these properties mostly reside in the fatty acid. We have shown here that the protein moiety can have a profound effect on the aggregation state of OA and, in practice, that the added protein can enhance the water solubility of the inherently toxic fatty acid (*54*, *55*) (see Figure 6 in Knyazeva et al. (*42*)). We are inclined, therefore, to interpret the results of this study as indicating that the protein can serve mainly as a carrier of the fatty acid, in agreement with our previous proposal (*40*).

Recently, it was shown that the cytotoxicity of BAMLET does not involve a classic apoptosis mechanism nor autophagy but rather that these complexes kill tumor cells via a mechanism involving lysosomal membrane permeabilization and consequent leakage of cathepsins (*29*). Also HAMLET interacts with lipid membranes perturbing their structure and integrity, and this interaction requires the participation of the fatty acid (*74*). Since it has been reported that OA is a membrane penetration enhancer by determining an increased conformational flexibility of membrane lipids (*75*), it could be well that a HAMLET-like complex can act as a delivery system of OA by enhancing its solubility in an aqueous environment, thus facilitating and enhancing its fluidizing or disrupting effect on lipid membranes.

There are recent data that possibly can be mentioned in support of the effects of OA in cancer therapy. It appears that monounsaturated fatty acids such as OA may influence breast cancer risk, since in southern European populations the intake of OA deriving from the use of olive oil appears to lead to a protective effect. Different mechanisms for the modulatory actions of olive oil, the richest dietary source of OA, on cancer have been proposed (*76*). It has been shown that OA influences the membrane structure and function (*77*, *78*) and, in particular, changes the membrane fluidity (*79*), resulting in the modulation of several enzymatic process, signal transduction pathways, as well as different stages of carcinogenesis (*76*). It has been reported that OA suppresses Her-2/neu overexpression, which in turn interacts synergistically with anti-Her-2/neu immunotherapy by promoting apoptotic cell death of breast cancer cells with Her-2/neu oncogene amplification (*80*). A genomic explanation connecting cancer with a diet rich in olive oil has been given (*81*). The protective effect of OA seems to operate also in various types of human malignancies previously shown to be influenced by olive oil consumption, including breast, ovarian, and stomach carcinomas (*76*). Therefore, this previously unrecognized property of OA seems to indicate that this fatty acid may regulate the malignant behavior of cancer cells (*82*). If the proposal/hypothesis of the protective role of OA in cancer is correct, we can envisage that the results here reported will be useful for a better understanding of the molecular details of protein/OA complexes and for delineating novel strategies of using OA as an anticancer agent.

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SUPPORTING INFORMATION AVAILABLE

Figures S1−S12. This material is available free of charge via the Internet at http://pubs.acs.org.

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