Purification and Structural Characterization of the Central Hydrophobic Domain of Oleosin*

Received for publication, March 20, 2002, and in revised form, July 10, 2002
Published, JBC Papers in Press, July 17, 2002, DOI 10.1074/jbc.M202721200

Ming Li‡‡, Denis J. Murphy§§, Ka-Ho K. Lee¶, Reginald Wilson**, Linda J. Smith**,
David C. Clark***‡‡, and Jao-Yiu Sung‡

From the ‡Department of Medicine & Therapeutics, 9/F, Clinical Building, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, New Territory, Hong Kong, §Cambridge Laboratory, John Innes Canter, Norwich NR4 7UH, United Kingdom, the ¶Department of Anatomy, Chinese University of Hong Kong, Shatin, Hong Kong, and the **Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, United Kingdom

The oil bodies of rapeseeds contain a triacylglycerol matrix surrounded by a monolayer of phospholipids embedded with abundant structural alkaline proteins termed oleosins and some other minor proteins. Oleosins are unusual proteins because they contain a 70–80-residue uninterrupted nonpolar domain flanked by relatively polar C- and N-terminal domains. Although the hydrophilic N-terminal domain had been studied, the structural feature of the central hydrophobic domain remains unclear due to its high hydrophobicity. In the present study, we reported the generation, purification, and characterization of a 9-kDa central hydrophobic domain from rapeseed oleosin (19 kDa). The 9-kDa central hydrophobic domain was produced by selectively degrading the N and C termini with enzymes and then purifying the digest by SDS-PAGE and electrophoresis. We have also reconstituted the central domain into liposomes and synthetic oil bodies to determine the secondary structure of the domain and its surface area. The spectra obtained from CD and FTIR were analyzed with reference to structural information of the N-terminal domain and the full-length rapeseed oleosin. Both CD and FTIR analysis revealed that 50–63% of the domain was composed of β-sheet structure. Detailed analysis of the FTIR spectra indicated that 80% of the β-sheet structure, present in the central domain, was arranged in parallel to the intermolecular β-sheet structure. Therefore, interactions between adjacent oleosin proteins would give rise to a stable β-sheet structure that would extend around the surface of the seed oil bodies stabilizing them in emulsion systems. The strategies used in our present study are significant in that it could be generally used to study difficult proteins with different independent structural domains, especially with long hydrophobic domains.

Storage lipids are found in the seeds of most higher plants. These are typically present as triacylglycerol-containing bodies of 0.5–2 μm diameter bound by a monomolecular phospholipid annulus into which is embedded a layer of specific amphipathic proteins, the oleosins (2–4). This storage oil serves as a carbon source and energy reserve that is mobilized following seed germination. In some plant species, such as rapeseed and sunflower, the seed oil content can be as high as 40–60% fresh weight, and oleosins constitute 10–20% of the total seed protein (5). Oleosins were initially defined as seed-specific proteins, associated exclusively with the surfaces of storage oil bodies in plants. The precise function and structure of oleosins have still not been elucidated. It is believed that they are responsible for the maintenance of a population of discrete small diameter oil bodies found in seed tissues, particularly during the dehydration process that accompanies seed maturation in most plant species (6, 7). In the absence of such stabilizing entities, the oil bodies would tend to coalesce during water removal to form an amorphous mass of lipid, which would have a very low surface area/volume ratio (8). This would severely impede the action of the lipases in mobilizing storage lipids following seed germination, because lipases are interfacial enzymes, whose activity is dependent on the available surface area of substrates (9).

Amino acid data derived from the studies of more than 40 different seed oleosins revealed that oleosin possesses a characteristic central hydrophobic domain containing ~70 uninterrupted and uncharged residues. This is the longest hydrophobic domain that has yet been found in any naturally occurring proteins. The central hydrophobic domain is flanked by relatively polar C-terminal and N-terminal domains. These domains are very diverse in their amino acid compositions. Oleosins are amphipathic proteins but they do not behave like bilayer membrane proteins, because they are localized at a single lipid/water interface between the triacylglycerol core of the oil body and the surrounding aqueous cytoplasm. Oleosins therefore have some analogies with other interfacial or monolayer-associated proteins, such as apolipoproteins, that are involved in lipid transport of mammalian circulatory systems (1) and hydrophobins found in fungi (10). Oleosins appear to act as a natural emulsifying and stabilizing agent at an oil/water interface. This suggests a possible biotechnological application for oleosins in the stabilization of emulsion systems, in industries such as food processing, pharmaceutical manufacture, and oil spillage treatment. In addition, oleosins have recently been proposed as a carrier for the expression and purification of recombinant pharmaceutical peptides and industrial enzymes (11, 12). Therefore, there is considerable interest in under-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 852-26332023; Fax: 852-26373852; E-mail: kgh7671@mailserv.cuhk.edu.hk.
‡‡ Present address: DMV International, NCB-Laan 80, PO Box 13, 5460 BA Vegnem, The Netherlands.

37888 This paper is available on line at http://www.jbc.org
standing the secondary structure of oleosin and how oleosin interacts with oil bodies.

We have studied previously (13) the secondary structure of a full-length 19-kDa oleosin protein from rapeseed. In addition, we have also examined a recombinant protein corresponding to the 6-kDa polar N-terminal domain of sunflower oleosin (14). These studies were performed using CD and Fourier transform infrared (FTIR) spectroscopy. The emerging model for the structure of oleosin is that of a central hydrophobic domain (mostly made up of extended β-structure) probably embedded into the lipidic core of oil bodies in vivo. However, the structure of the central domain remains unclear due to difficulties involved in producing and purifying the central domain. We have tried to express this central domain in Escherichia coli, in yeast, and in cell-free translation systems. These attempts were unsuccessful because the central domain was highly hydrophobic and produced extremely low yields. In contrast, the secondary structures that are located on the surface of oil bodies and may also extend out from the surface of the oil bodies (13, 14). Although most investigators accept the general feature of this model, it has been suggested that the central hydrophobic domain could contain a hairpin loop. This loop is centered on three conserved proline residues that form an anti-parallel β-sheet structure (3), rather than forming a single parallel β-sheet structure as proposed in our original model (13). Furthermore, studies on safflower and sunflower oil bodies have led some researchers to suggest that the central domain was composed of mainly α-helices (15). However, these models have not yet been verified by experimental evidence because it is difficult to produce and reconstitute pure central hydrophobic domain of oleosin.

Therefore, the aims of the present study are to generate and purify the central hydrophobic domain and to elucidate the secondary structure of liposome or the oil bodies-reconstituted central domain by CD and FTIR. In many proteins that contain structurally distinct regions, the different domains fold independently to form separate but linked units of the protein. Hence, the structure of individual protein domains may be studied in isolation to circumvent some substantial difficulties (16–18). This allows for the unequivocal assignment of particular structures to particular domains. Because oil bodies from rapeseed were difficult to purify to homogeneity and the oleosin obtained from these purified natural oil bodies was always contaminated with other proteins as demonstrated by SDS-PAGE, the natural oil bodies were corrupted, and the intact oleosin protein was purified by SDS-PAGE coupled with electroelution. This purified oleosin was used in subsequent reconstitution, selective enzyme digestion, and secondary structure determination in this study. Equally importantly, the use of a reconstituted system, resembling as much as possible the in vivo system, allows for the highest probability that proteins are folded into a native conformation. The results presented here show that the purified central hydrophobic domain is mostly made up of an extended parallel β-sheet with relatively minor amounts of anti-parallel structure. The significance of these findings for oleosin-oil body interactions is discussed.

EXPERIMENTAL PROCEDURES

Materials

Oleosins were obtained from mature seeds of Brassica napus. The lipid l-α-phosphatidylcholine type IX-E from egg yolk and trioleoylglycerol were obtained from Sigma, as was protease K. All other chemicals were of analytical grade.

Purification of 19-kDa Oleosin

Oil body membrane proteins were purified from rapeseeds as described previously (13). An equal volume of 2× SDS sample buffer was added to the resuspended oil body membrane proteins. The sample was then boiled for 5 min, vortexed briefly, and loaded onto a preparative SDS-PAGE gel containing 12% acrylamide (19). The gels were rinsed with double distilled H2O, and a strip of the gel was cut with a razor for staining with Coomassie Blue R-250. Gel slices, containing the 19-kDa protein, were localized by Western blotting, and the corresponding areas of the original gel were excised for electrodetection. The eluting buffer consisted of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM CaCl2, 0.2% SDS, and 1 mM EDTA. The purity of the eluted oleosin was checked by analytical SDS-PAGE (18, 19). The eluted 19-kDa oleosin was precipitated by adding 2.5–3 volumes of ice-cold acetone and mixing thoroughly. The acetone/eluicate mixture was allowed to precipitate for 2 h at −80 °C. The tubes were then centrifuged at 20,000 × g for 30 min at 4 °C, the acetone supernatant poured off, and the tubes inverted to drain. The precipitate was then solubilized in 6 μg guanine HCl, 0.1 mM Tris-HCl buffer (pH 8.0), 1 mM dithiothreitol, and 1 mM EDTA. The dissolved protein sample was placed into a dialysis tube and dialyzed against refolding buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM CaCl2, 10 mM sodium cholate, and 1 mM EDTA for 72 h. The buffer was changed 4 times at 4 °C.

Reconstitution of 19-kDa Oleosin into Artificial Oil Bodies

Lipid films were prepared by drying 20 mg of phospholipids in chloroform under a stream of nitrogen, followed by lyophilization overnight. Solvent-free phosphatidylcholines were solubilized in a small volume of sodium cholate made up in phosphate-buffered saline (PBS) (22). The preparation was mixed vigorously until a clear solution was obtained with a final phosphatidylcholine concentration of 20 mg/ml. A greater than 4-fold excess (w/w) of sodium cholate was used to dissolve the phospholipid. The concentration of cholate was confirmed to be above its critical micellar concentration of 7 mM. The purified and refolded oleosin protein in refolding buffer and trioleoylglycerol was then added to produce a final phospholipid/oil/polypeptide ratio by weight of 1:20:2. The solution was sonicated for 5 min to form a uniform suspension. The suspension was dialyzed against 5 changes of buffer (30 mM Tris-HCl (pH 7.4), 1 mM EDTA) and followed by sonication for 5 min. The phospholipid/oil/protein suspension was then centrifuged for 30 min at 5,000 × g. The reconstituted oil bodies were collected by flotation and washed in refolding buffer to remove oil-free proteins. Finally the oil bodies were resuspended in 10% sucrose made up in 30 mM Tris-HCl (pH 7.4). The mixtures were again sonicated for 30 s and allowed to stand for 2 h. The reconstituted phospholipid-oil-oleosin complexes were then analyzed by immunocytochemistry and FTIR spectroscopy.

Electron Microscopy

Reconstituted oil body preparations were reconstituted in LMP-agarose. The preparation was fixed in glutaraldehyde and embedded in LR white resin as described previously (21). Sections of 100 nm thickness were cut on a MT6000 Ultramicrotome and mounted on to nickel grids (300 mesh). The grids were submersed for 40 min in a solution of 150 mM NaO buffer, pH 7.0, 0.1% Triton X-100, and 0.1% BSA. The grids were then incubated with anti-oleosin polyclonal rabbit antibodies (diluted 1:1000 in PBS buffer, 0.1% Triton X-100, and 0.1% BSA) for 1 h. The grids were washed 4 times in PBS, 0.1% Triton X-100, and 0.1% BSA, and then incubated for 1 h in goat anti-rabbit IgG conjugated with 12 nm colloidal gold particles (diluted 1:40 with PBS, 0.1% Triton X-100, and 0.1% BSA). The grids were post-stained for 15 min in 1% aqueous uranyl acetate and 2 min in Reynold’s lead citrate. The sections were examined on a Philips 400 transmission electron microscope.

Preparation of the Central Hydrophobic Domain of Oleosin

The 9-kDa central domain was prepared by treating purified full-length 19-kDa rapeseed oleosin, reconstituted into phospholipid/oil bodies as described above, with 0.1 mg/ml protease K for 4 h at 25 °C. This treatment resulted in the preferential digestion of the hydrophilic N- and C-terminal domains. The lipid-embedded central domain was inaccessible to the protease (22, 23). The digested oil body samples were then centrifuged at 3000 × g for 10 min, washed, and resuspended in 10% sucrose (prepared in Tris-HCl (pH 7.4)). The identity of the rapeseed central domain polypeptide that remained trapped in the purified oil bodies was verified by sequencing of the first 12
residues from its N terminus. The secondary structure of the central domain (trapped in oil bodies and produced by selective protease digestion) was determined by FTIR, as dry films and in aqueous medium. The spectra for test samples were compared with those obtained for the pure oil (control) to identify the bands obtained from the central peptide. The spectra presented are derived from an average of 5 scans recorded at 10 nm/mm. Instrument sensitivity of ± 20 millidegrees full scale was routinely used, along with a 4 s time constant (24). The instrument was regularly calibrated using ammonium D-10-camphorsulfonate (25) and p-nitroaniline (26).

Circular Dichroism Spectroscopy

CD spectra were obtained using a Jasco J-600A spectropolarimeter (wavelength range 260–185 nm), under constant nitrogen flush and using 1 mm cells at 20 °C. The data were recorded on-line using an IBM computer. The spectra presented are derived from an average of 5 scans recorded at 10 nm/mm. Instrument sensitivity of ± 20 millidegrees full scale was used. The complex solution contained liposome-reconstituted (0.1 mg/ml) complex solution (51). The complex solution contained liposome-reconstituted (0.1 mg/ml) complex solution containing no protein. The CD instrument was regularly calibrated using ammonium D-10-camphorsulfonate (25) and p-nitroaniline (26).

Fourier Transform Infrared Spectroscopic Studies

The FTIR spectra for the central domain of oleosin and intact 19-kDa oleosin (in either artificial oil bodies or liposomes) were measured at room temperature on a Bio-Rad FTS60 spectrometer, using a liquid nitrogen-cooled mercury cadmium telluride detector (resolution of 2 cm⁻¹) and Win-IR software. Triangular apodization was employed. The samples were introduced to the spectrometer in solution form. A total of 1200 scans was collected for each spectrum to get a reasonable signal/noise ratio. Difference spectra were obtained by digitally subtracting solvent spectra and phospholipid/oil body (containing no protein) spectra. Each sample solution was determined in three batches. The individual spectrum of three determinations for each sample was obtained and averaged to produce a single spectrum. The data were processed using GRAMS/32 (Galactic Industries). Second derivative spectral analysis and Fourier self-deconvolution were used to locate the position of the overlapping components of the amide I band, to confirm band center frequencies and assign them to different secondary structures. Secondary structure components were accomplished by least squares iteration. Gaussian band shapes were assumed for the deconvoluted bands. The amide I band (1600–1700 cm⁻¹) was deconvoluted into three components: (i) 30% N-H bend and 40% C–N stretch, near 1550 cm⁻¹, and III (40% C–N stretch, 30% N–H bend, near 1300 cm⁻¹) are expected to occur at 1670.0–1663.0 cm⁻¹. The type II β-turn vibrations, which can be assigned to the band near 1640.0 cm⁻¹ and 1530.0–1550.0 cm⁻¹, are normally assigned to turn structures (57). In addition, the band at 1660.0–1663.0 cm⁻¹ (normally assigned to turn structures) is often found to reflect the presence of some helical or irregular structures. Unordered Structures—Although it is difficult to assign a particular sub-region of the amide I region to unordered structures, unordered structure is generally assigned to the band near 1640.0–1651.0 cm⁻¹ in the IR (48). The 1654.0–1675.0 cm⁻¹ region appears to characterize unordered structure on the basis of IR studies of feather keratin and denatured proteins (58). The characteristic vibrations for a protein containing mixed domains of extensive β-turn and less extensive β-sheet structures, from well established β-sheet vibrations and newly established β-turn vibrations, are expected to occur at 1670.0–1680.0 and around 1240.0 cm⁻¹ (57).

RESULTS

Purification of Full-length Oleosin Protein

Oleosins are relatively abundant in rapeseed, constituting 8–20% of the total seed protein (13, 14). Therefore, mature rapeseeds were used to produce the full-length 19-kDa oleosin protein in our study. Rapeseed oil bodies are difficult to purify to homogeneity and are frequently contaminated with other proteins. In fact, when we used the proteins obtained from thoroughly washed and purified natural oil bodies, there were always more than 4 protein bands present in our SDS-PAGE. Hence, we used purified 19-kDa oleosin and reconstituted the protein into liposomes or synthetic oil bodies instead of using...
natural intact oil bodies. Oleosin contains highly hydrophobic domain, and once removed from oil bodies, they tend to aggregate in the absence of solubilizing detergents. Purification of full-length rapeseed oleosin was attained by preparative SDS-PAGE on gradient gels containing 10–18% acrylamide, followed by electroelution. The purity of the 19-kDa rapeseed oleosin is shown in Fig. 1A. The results show that a small amount of dimeric 19-kDa oleosin is always present at the level of the 40-kDa protein marker. There was also a trace of a putative trimer at 60 kDa. These bands were formed by oligomers of the 19-kDa oleosin because (a) the sample originated from a single 19-kDa band excised from an SDS-PAGE gel, and (b) the 40- and 60-kDa bands were recognized by antibodies specific to the 19-kDa oleosin (results not shown). The purified 19-kDa oleosin protein was completely denatured by adding 6 M guanidine hydrochloride and then refolded by dialyzing against refolding buffer. The appearance of the CD and FTIR spectra suggested that the central domain has a well ordered protein structures. Good structural consistence among different repeats of the refolding processes as assayed by CD and FTIR methods indicates that the polypeptides were refolded into ordered structures.

Reconstitution of Oleosins into Artificial Oil Bodies

In the absence of oleosin, newly synthesized phospholipid/trioleoylglycerol oil bodies were found to be unstable in 10% sucrose, 50 mM Tris-HCl (pH 7.4). Within a matter of a few minutes, the oil bodies aggregated to form an amorphous lipidic mass. In contrast, suspensions containing oleosin-phospholipid-trioleoylglycerol oil body complex were stable and maintained turbidity for more than 10 h at 4 °C. This reconstituted artifical oleosin-oil body complex contained a population of spherical oil bodies with sizes ranging from about 0.4 to 1.6 μm, which is similar to the size of native oil bodies in vivo (Fig. 2A). However, oil bodies containing only the central domain of oleosin were almost as unstable as oil bodies containing no protein. This was demonstrated by a decrease in turbidity of the reconstituted oil body suspensions and by electron microscopy (Fig. 2B). These data indicate that stability of oleosin/oil body emulsion is not conferred by one protein domain alone. Rather, it is through the interaction of several domains, both within and between oleosin molecules, that enables them to maintain the integrity of the oil body during prolonged storage. The reconstituted oil body preparations were examined immunohistochemically. Antibodies specific against the 19-kDa rapeseed oleosin was used to detect the reconstituted oleosin. It was found that the 19-kDa oleosins were localized on the surface of the reconstituted oil bodies, as indicated by the dark spots around the surface of the artificial oil body (Fig. 2, C and D). It was also demonstrated that the antigen sites of 19-kDa oleosin were in the sequences of N/C-terminal domains.

The 9-kDa central domain was prepared from its full-length oleosin as described under “Experimental Procedures.” The purity of the 9-kDa central domain is shown in Fig. 1A. The preparation was more than 95% pure, with only minute traces of the uncleaved 19-kDa oleosin. The purity of the 9-kDa central domain is shown in Fig. 1. The structure of the 9-kDa polypeptide, trapped in the oil bodies, was studied by FTIR. The recovered 9-kDa central domain was also deprived of oil, dissolved in 6 M guanidine hydrochloride, and reconstituted into liposomes. The structure of the liposome reconstituted 9-kDa central domain was studied by CD. The CD and FTIR spectra suggested that the central domain has a well ordered protein structure. The structural consistency among different repeats of the process indicated that the polypeptide folded into ordered structures.

Estimation of Secondary Structure Content by CD

Attempts to obtain CD spectra from reconstituted oleosin/oil body were unsuccessful due to the turbidity of the solution. Therefore, the CD spectra of liposome-reconstituted central domain (Fig. 3) were plotted against the spectra from liposome-
Conformational Assignments of Components from FTIR Spectra of Reconstituted Central Domain and Intact 19-kDa Oleosin

Full Range Spectra—The full range deconvolution FTIR spectra (1,000–1,800 cm$^{-1}$) for reconstituted rapeseed oleosin central domain and intact 19-kDa oleosin are shown in Fig. 4, A and B. The corresponding spectrum for the reconstituted sunflower N-terminal domain has been published previously (14).

Central Domain—The main amide I peaks of the central domain occurred at 1616.0, 1627.2, 1640.0, 1653.0, and 1673.5 cm$^{-1}$, with shoulder at 1686.2 cm$^{-1}$ (Fig. 4, A and C). The main amide II peaks were found at 1523.5, 1534.0, 1551.0, and 1580.5 cm$^{-1}$, together with a smaller peak at 1512.0 and 1566.0 cm$^{-1}$. The main amide III peak occurred at 1251.9 cm$^{-1}$ and a small peak at 1268.7 cm$^{-1}$. The peaks at 1616.0, 1627.2, 1640.0, 1523.5, 1534.0, and 1551.0 cm$^{-1}$ and the shoulder at 1686.2 cm$^{-1}$ are indicative of $\beta$-sheet structure. The peak at 1653.0 cm$^{-1}$ is probably unordered structure, overlapped with type I or III, $\beta$-turn, or $\alpha$-helical structures. This is because there is often overlap of $\alpha$-helical absorption with that of random coil and type I and III $\beta$-turn structures. The shoulder at 1686.2 cm$^{-1}$ and the peak at 1627.2 cm$^{-1}$ indicates the presence of intermolecular $\beta$-sheet structure, forming a protein oligomer shell (52, 53). The peaks at 1627.2, 1640.0, and 1551.0 cm$^{-1}$ and shoulder at 1534.0 cm$^{-1}$ are indicative of parallel $\beta$-sheet structure. The peaks at 1616.0 and 1512.0 cm$^{-1}$ are indicative of anti-parallel $\beta$-strand structure, overlapped with tyrosine absorption. The presence of intermolecular and parallel $\beta$-sheet structures infers that the central domain of oleosin forms a protein oligomer shell to stabilize plant triglyceride droplets. The secondary structure contents of the reconstituted central domain, determined by FTIR analysis, was found to be similar to calculations derived from the CD analysis. In both cases, the major component is $\beta$-sheet structure, which is estimated to contribute 63% (FTIR data) to 50% (from CD data), of the total central domain, with a very minor 5–7% $\alpha$-helical structure. The 63% of $\beta$-sheet structure, determined by FTIR, can be further subdivided into 51% parallel with intermolecular $\beta$-sheet structure and 12% anti-parallel structures.

Full-length 19-kDa Oleosin—The main amide I peaks for the full-length 19-kDa oleosin occurred at 1616.5, 1634.0, and 1650.0 cm$^{-1}$, with shoulders at 1670.0, 1680.5, and 1692.0 cm$^{-1}$ (Fig. 4, B and D). The main amide II peaks occurred at 1511.5, 1522.5, 1547.0, and 1584.0 cm$^{-1}$, together with a shoulder at 1536.5 cm$^{-1}$. The main amide III peak was found at 1254.5 cm$^{-1}$ with small peaks at 1278.5 and 1300.0 cm$^{-1}$. The peaks at 1650.0, 1278.5, and 1300.0 cm$^{-1}$ were indicative of $\alpha$-helical structures probably overlapped with unordered structures. The peaks at 1616.5 and 1634.0 cm$^{-1}$ and a shoulder at 1692.0 cm$^{-1}$ were indicative of $\beta$-sheet structure. The shoulder at 1692.0 and the peaks at 1634.0, 1536.5, and 1557.0 cm$^{-1}$ indicate the presence of parallel and intermolecular $\beta$-sheet structures, forming a protein oligomer shell (60, 61). The peaks at 1616.5 and 1511.5 cm$^{-1}$ were indicative of anti-parallel $\beta$-strand structure, overlapped with tyrosine absorption. The presence of parallel and intermolecular $\beta$-sheet structures strongly suggests that the full-length 19-kDa oleosin forms a protein oligomer shell in its reconstituted form. The structural contents of the reconstituted 19-kDa oleosin, as calculated from the FTIR spectra, were similar to calculations derived from CD analysis. $\beta$-Sheet structures were determined to be the major component (48%) of the 19-kDa oleosin. The remainder was made up of 16% $\alpha$-helical, 15% $\beta$-turn, and 21% unordered structures.

**DISCUSSION**

The oleosin molecule, based on its primary amino acid sequence, can be divided into three distinct structural domains as follows: (a) a relatively polar N-terminal domain containing about 50 residues; (b) a central hydrophobic domain with about 75 residues; and (c) a polar/amphipathic C-terminal domain with about 65 residues (13, 14). To date, the protein sequence of more than 40 seed-specific oleosins, from different plant species, have been described. These studies revealed that all oleosins have a similar domain structure. In particular, they
share a highly conserved hydrophobic central domain containing about 75 residues, flanked by relatively polar/amphipathic N- and C-terminal domains. These terminal domains are quite variable in length and composition (2, 3, 63). Indeed, it has been shown recently (64) that the 19-kDa rapeseed oleosin protein can be engineered to contain an additional C-terminal polypeptide (β-glucuronidase, 68 kDa) to create a chimeric 87-kDa fusion protein. In transgenic plants expressing this recombinant fusion protein, the protein is able to associate with oil bodies and stabilize them in the same manner as unmodified 19-kDa oleosin.

Previous physical studies (13, 14) in our laboratory, using FTIR and CD spectroscopy, have indicated that the three major 19-kDa oleosin isoforms purified from rapeseed each contain 40–50% β-sheet structures. The finding agrees with predictions of the secondary structure based on the primary sequences of the protein (64). These predictions indicate that the central hydrophobic domain, which makes up 40% of the total protein, is likely to be composed of mostly β-sheet structure. It is possible that this hydrophobic β-sheet is extended in a linear conformation, which will allow it to interact with neighboring oleosin molecules to form oligomeric β-sheet structures. Indeed, analogous oligomeric associations have been found in other interfacial amphipathic proteins, such as the fungal hydrophobins (10). Alternatively, the hydrophobic central domain may contain a hairpin loop formed around three conserved proline residues. This allows the linear β-sheet to double back upon itself to form an anti-parallel β-sheet structure (3).
would extend from the surface deep into the hydrophobic interior of the oil bodies. Previous studies have not been able to differentiate between these two alternatives.

In order to resolve the finer structures of the central domain of oleosin, it is necessary to reconstitute the purified central domain preparations into oil bodies resembling as closely as possible those in vivo. The examination and comparison of the secondary structures of the full-length oleosin and the N-terminal and central domains serve two purposes. First, it reveals the interaction between the various domains and the oil bodies and their physical behavior (e.g. the stability of the resultant emulsions). Second, the analysis of discrete protein domains allows for a more definite assignment of particular secondary structures to a given domain than is possible when an entire protein is analyzed. Our results indicate that maximum stability of reconstituted oil body emulsions is only possible with the intact oleosin protein. Once the N- and C-terminal domains were removed by protease digesting, the resulting rapeseed 9-kDa central domain was a relatively poor emulsifying and stabilizing agent. This suggests that the surface-oriented, amphipathic N- and C-terminal domains may play an important role in emulsion formation.

The data presented in this study indicated that when the peripheral N- and C-terminal domains of the rapeseed oleosin were removed, the residual 9-kDa central hydrophobic domain that remained contained 50–63% β-sheet structure. This 9-kDa polypeptide contained the entire 75-residue hydrophobic domain (strongly predicted to be β-structure), plus about 15 residues of the amphipathic C-terminal domain (predicted to be α-helical) (63). Detailed analysis of the FTIR spectra allows us to identify the existence of intermolecular β-sheet structure and to distinguish between the parallel and anti-parallel types of β-sheet structure, using assignments described previously (47–49, 60, 61). This shows that, of the 63% total β-sheet structure determined by FTIR spectroscopy, some 51% were arranged in parallel with intermolecular β-sheet structure, and only 12% were anti-parallel. Therefore, the three universally conserved proline residues, found within the “proline knot” motif of the oleosin central domain, were not likely to be important for determining the topology of oleosin as predicted previously (3). It has been reported from other studies (66) that when the three proline residues were substituted by leucine, the proline knot was required for oil body targeting and was less important for determining the topology of the central domain. It was also possible that a small part (~12%) of the anti-parallel β-sheet structure, a hairpin loop formed around three conserved proline residues, is contained in the hydrophobic central domain.

Our findings make it difficult to reconcile with speculations that virtually the entire central domain is structured as a “hairpin loop,” composed mainly of anti-parallel β-sheet structure (3). In addition, the entire central domain that forms hairpin loop should not contain intermolecular β-sheet structure (3). The proposed heterodimers formed by oleosins (63, 67, 68) are more likely to be stabilized by the association of parallel β-sheets to form a β-sheet hairpin. In a hairpin loop structure, most of the hydrogen bond-forming potential of the protein would be satisfied via anti-parallel β-sheet. This would make the formation of dimers and intermolecular β-sheet structure less likely. We have commented previously on the tendency for purified oleosins to self-associate to form dimers, trimers, and higher order oligomers (13). Presently, we confirmed the existence of the dimeric and trimeric forms of oleosin. Such self-associations have also been observed when a single oleosin isoform from soybean was expressed in transgenic rapeseed plants. Hence, identical oleosin molecules can interact to form homo-oligomers, some of which remain associated even in the presence of strong denaturants, such as SDS. This indicates that oleosins, both in vitro and in vivo, can self-associate. We propose that this occurs via hydrogen bonding between the adjacent parallel β-sheet found in the uncharged central domain. This would form a tightly knitted β-sheet structure that would extend around the entire surface of the oil body, forming a proteinaceous cage that encapsulates and stabilizes the storage lipids within.

Our results also indicated that maximum stability of reconstituted oil body emulsions was only attained by reconstitution of the intact oleosin with oil bodies. The 9-kDa central domain was a relatively poor emulsifying and stabilizing agent. The surface-oriented N- and C-terminal domains play an important role in emulsion formation. It is also likely that N- and C-terminal domains can associate with each other to form dimers, trimers, or oligomers, which would form a tightly knotted amphipathic structure extending around the entire surface of the oil body. The amphipathic nature of N- and C-terminal domains together with the central domain would form a proteinaceous cage that stabilizes the storage lipids within. This is required for the maintenance of a population of discrete small-medium size oil bodies.

REFERENCES
1. Murphy, D. J. (1990) Progr. Lipid Res. 29, 299–324
2. Murphy, D. J. (1993) Prog. Lipid Res. 32, 247–260
3. Huang, A. H. C. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 177–200
4. Napier, J. A., Stobart, A. K., and Shewry, P. R. (1991) Plant Mol. Biol. 19, 945–956
5. Murphy, D. J., and Cummins, I. (1989) Phytochemistry 28, 2063–2069
6. Hills, M. J., Watson, M. D., and Murphy, D. J. (1993) Planta 189, 24–29
7. Murphy, D. J., Ross, J. H. E., and Prickett, H. W. (1995) in Plant Lipid Metabolism (Kader, J. C., and Mazliak, P., eds) pp. 558–560, Kluwer Academic Publishers Group, Dordrecht, Netherlands
8. Cummins, I., Zhills, M. J., Ross, J. H. E., Hobbs, D. H., Watson, M. D., and Murphy, D. J. (1994) Plant Mol. Biol. 23, 1015–1027
9. Mukherjee, K. D., and Hills, M. J. (1993) in Lipases:Their Structure, Biochemistry, and Application (Woolley, P., and Pattersen, S. B., eds) pp. 49–75, Cambridge University Press, UK
10. Wessels, J. G. H. (1996) Trends Plant Sci. 1, 9–14
11. Van Rooijen, G. J. H., and Moloney, M. M. (1995) Bio-Technology 13, 72–77
12. Parmenter, D. L., Boote, J. G., Van Rooijen, G. J. H., Yeung, E. C., and Moloney, M. M. (1995) J. Agric. Food Chem. 43, 1167–1180
13. Li, M., Smith, L. J., Clark, D. C., Wilson, R., and Murphy, D. J. (1992) J. Biol. Chem. 267, 8245–8253
14. Li, M., Keddie, J. S., Smith, L. J., Clark, D. C., and Murphy, D. J. (1993) J. Biol. Chem. 268, 17504–17512
15. Lacey, D. J., Wellner, N., Beaudoin, F., Napier, J. A., and Shewry, P. R. (1998) Biochem. J. 334, 469–477
16. Orban, J., Alexander, P., and Bryan, P. (1992) Biochemistry 31, 3604–3611
17. Zhang, Z., Ashton, R. W., Ni, F., and Scheraga, H. A. (1992) Biochemistry 31, 4425–4431
18. Rizko-Venoscic, M., Minard, P., Demadrid, M., and Yoon, J. M. (1995) Biochemistry 34, 16543–16551
19. Schagger, H., and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual (Nolan, C., ed) pp. 18.47–18.80, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Murphy, D. J., and Cummins, I. (1989) Plant Physiol. 80, 47–54
22.Ao, D. M. Y., Cummins, I., and Murphy, D. J. (1989) Biochm. Biophys. Acta 1005, 97–102
23. Rausuens, V., Kuyschaert, J. M., and Goormaghtigh, E. (1997) J. Biol. Chem. 272, 292–297
24. Clark, D. C., and Smith, L. J. (1989) J. Agric. Food Chem. 37, 627
25. Takakawa, T., Konno, T., and Meguro, H. (1975) Anal. Sci. 1, 215–218
26. Konno, T., Meguro, H., and Tanizuma, K. (1987) Anal. Biochem. 67, 226–232
27. Sreevama, N., Venayyin, S. Y., and Woody, R. W. (1999) Protein Sci. 8, 370–380
28. Greenfield, N. J. (1996) Anal. Biochem. 235, 1–10
29. Yang, J. T., Wu, C.-S. C., and Martinez, H. M. (1986) Methods Enzymol. 130, 208–269
30. Sreevama, N., Venayyin, S. Y., and Woody, R. W. (2000) Anal. Biochem. 287, 243–251
31. Chen, Y.-H., Yang, J. T., and Martnez, H. M. (1972) Anal. Biochem. 47, 442–447
32. Murphy, D. J., and Cummins, I. (1989) Plant Physiol. 100, 97–102
33. Sreevama, N., Venayyin, S. Y., and Woody, R. W. (2000) Anal. Biochem. 287, 243–251
34. Forysthe, G. E., Malcolm, M. A., and Moler, C. B. (1977) Computer Methods for

2 C. Sarmiento, E. Herman, and D. J. Murphy, unpublished results.
35. Renugopalakrishnan, V., Rapaka, R. S., Collette, T. W., Carreira, L. A., and Bhatnagar, R. S. (1985) *Biochem. Biophys. Res. Commun.* **126**, 1029–1035

36. Arrondo, J. L. R., and Gonçalves, F. M. (1999) *Prog. Biophys. Mol. Biol.* **72**, 367–405

37. Goormaghtigh, E., and Ruysschaert, J. M. (1990) in *Molecular Description of Biological Membrane Components by Computer-aided Conformational Analysis* (Brasseur, R., ed) pp. 285–329, CRC Press, Inc., Boca Raton, FL

38. Vigneron, L., Ruysschaert, J. M., and Goormaghtigh, E. (1995) *J. Biol. Chem.* **270**, 17685–17696

39. Abbott, T. P., Wolf, W. J., Wu, Y. V., Butterfield, R. O., and Kleiman, R. (1991) *Appl. Spectrosc.* **45**, 1665–1673

40. Lee, D. C., Haris, P. I., Chapman, D., and Mitchell, R. C. (1990) *Biochemistry* **29**, 9185–9193

41. Dousseau, F., and Pezolet, M. (1990) *Biochemistry* **29**, 8771–8779

42. Sarver, R. W., and Krueger, W. C. (1991) *Anal. Biochem.* **199**, 61–67

43. Pelton, J. T., and McLean, L. R. (2000) *Anal. Biochem.* **277**, 167–176

44. Kabsch, W., and Sander, C. (1983) *Biopolymers* **22**, 2577–2637

45. Parker, F. S. (1983) *Application of IR, Roman and Resonance Roman Spectroscopy in Biochemistry*, pp. 349–398, Plenum Publishing Corp., New York

46. Krimm, S., and Dwivedi, A. M. (1982) *Science* **216**, 407–408

47. Chirgadze, Yu. N., and Nevskava, N. A. (1976) *Biopolymers* **15**, 607–625

48. Pelton, J. T., and McLean, L. R. (2000) *Anal. Biochem.* **277**, 167–176

49. Chirgadze, Yu. N., and Nevskava, N. A. (1976) *Biopolymers* **15**, 627–636

50. Susi, H., and Byler, D. M. (1983) *Biochem. Biophys. Res. Commun.* **115**, 391–397

51. Chu, H. L., Liu, T. Y., and Lin, S. Y. (2001) *Aquat. Toxicol.* **55**, 171–176

52. Kabsch, W., and Sander, C. (1983) *Biopolymers* **22**, 2577–2637

53. Renugopalakrishnan, V., Hozowitz, P. M., and Glimcher, M. J. (1985) *J. Biol. Chem.* **260**, 11406–11413

54. Lagant, P., Vergoten, G., Fleurry, G., and Loucheux-Lefevre, M. H. (1984) *Eur. J. Biochem.* **139**, 137–148

55. Lagant, P., Vergoten, G., Fleurry, G., and Loucheux-Lefevre, M. H. (1984) *Eur. J. Biochem.* **139**, 149–154

56. Seaton, B. A. (1983) *Biophysical Studies of Protein Conformation*, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, MA

57. Ishizaki, H., Balaram, P., Nagaraj, R., Venkatachalapathy, Y. V., and Tu, A. T. (1981) *Biophys. J.* **36**, 509–517

58. Koening, J. L., and Tabb, D. J. (1980) in *Analytical Applications of FT-IR to Molecular and Biological Systems* (Durig, J. R., ed) pp. 241–255, D. Reidel Publishing Co., Boston, MA

59. Johnson, W. C., Jr. (1999) *Proteins Struct. Funct. Genet.* **35**, 307–312

60. Rahmelow, K., Hußner, W., and Ackermann, T. (1998) *Anal. Biochem.* **257**, 1–11

61. Lorenz, V. A., Villaverde, J., Trézéguet, V., Lauquin, G. J. M., Brandolin, G., and Padros, E. (2001) *Biochemistry* **40**, 8821–8833

62. Deleted in proof

63. Lee, K., Ratnayake, C., and Huang, A. H. C. (1995) *Plant J.* 7, 603–611

64. Van Rooijen, G. J. H., and Moloney, M. M. (1995) *Plant Physiol.* **106**, 1353–1361

65. Deleted in proof

66. Abell, B. M., Holbrook, L. A., Abenes, M., Murphy, D. J., Hills, M. J., Moloney, M. M. (1997) *Plant Cell* **9**, 1481–1493

67. Lee, K., and Huang, A. H. C. (1994) *Plant Mol. Biol.* **26**, 1981–1987

68. Tzen, J. T. C., Lai, Y.-K., Chan, K.-L., and Huang, A. H. C. (1990) *Plant Physiol.* **94**, 1282–1289