Annu. Rev. Entomol. 2000. 45:233–260

LIPID TRANSPORT BIOCHEMISTRY AND ITS ROLE IN ENERGY PRODUCTION

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Key Words lipophorin, adipokinetic hormone, apolipophorin III, diacylglycerol, receptor

■ **Abstract** Recent advances on the biochemistry of flight-related lipid mobilization, transport, and metabolism are reviewed. The synthesis and release of adipokinetic hormones and their function in activation of fat body triacylglycerol lipase to produce diacylglycerol is discussed. The dynamics of reversible lipoprotein conversions and the structural properties and role of the exchangeable apolipoprotein, apolipophorin III, in this process is presented. The nature and structure of hemolymph lipid transfer particle and the potential role of a recently discovered lipoprotein receptor of the low-density lipoprotein receptor family, in lipophorin metabolism and lipid transport is reviewed.

PERSPECTIVES AND OVERVIEW

For many biochemical processes and their regulation, insects provide a fascinating yet relatively simple model system. In the last few years, considerable progress has been made in a number of areas pertinent to lipid mobilization and transport in insects. The scope of this research encompasses regulation of the release of neurohormones (adipokinetic hormones, AKHs), the mechanism of action of fat body triacylglycerol (TAG) lipase, molecular details of hemolymph lipid transport, and cell surface receptors involved in endocytosis of lipoproteins.

Advances made in the area of the biosynthesis and structure of the multifunctional insect lipoprotein, high-density lipophorin (HDLp) are of particular interest. The functioning of this lipoprotein differs from those found in well studied, but complex, vertebrate systems. Indeed, it is the relative simplicity of the invertebrate system that offers possibilities for insight into processes pertinent to both systems. Flight-induced lipid loading onto HDLp is accompanied by association of multiple copies of the exchangeable apolipoprotein, apolipophorin III (apoLp-III).

0066-4170/00/0107-0233/$14.00

Because insect apoLp-III is the only apolipoprotein for which a complete three-dimensional structure is known, this system represents a unique model for investigating lipid-protein interactions.

In contrast to the functioning of lipophorin as a lipid shuttle in adult insects, during larval development endocytic uptake of HDLp is apparent. An endocytic receptor belonging to the low-density lipoprotein (LDL) receptor gene superfamily has recently been cloned, offering an exciting new avenue of research opportunity.

Long-distance flight of insects is a complex process that offers an attractive model for prolonged physical exercise. For instance, a pest insect such as the migratory locust is capable of uninterrupted flights of 10 h, covering more than 200 km. Although carbohydrate provides most of the energy during the initial period of flight, after 30 min, principally stored TAG is mobilized to power sustained flight. Consequently, the pivotal enzyme on which long-term flight of insects depends is fat body TAG lipase. The activity of this lipase seems to be controlled by AKH, which is released from the corpus cardiacum in response to flight activity. The enzyme catalyses the hydrolysis of the stored TAG to diacylglycerol (DAG), which is subsequently released into the insect blood and transported by lipophorin. Specific lipophorin subspecies involved in transporting DAG in hemolymph are believed to have receptor-mediated roles in flight, vitellogenesis and general lipid metabolism. Consequently, their primary sequence and properties are of great interest.

ADIPOKINETIC HORMONE-INDUCED LIPID MOBILIZATION

Release of Adipokinetic Hormones

Adipokinetic hormones (AKHs) comprise a family of structurally related N- and C-blocked small peptides. Despite their strong hydrophobicity, AKHs appear to be transported in hemolymph in their free form and not associated with a carrier protein (65). Structures of AKHs have been reported for many insects (33). Although many insects possess a single AKH species, three distinct AKHs have been identified in *Locusta migratoria*: a decapeptide AKH I and two octapeptides (AKH II and III), of which AKH I is by far the most abundant (62).

Details on AKH biosynthesis are particularly confined to a few locust species. The three AKHs of *L. migratoria* are synthesised as preprohormones (prepro-AKH), the amino acid sequences of which have been deduced from their cDNA sequences (14). All three preprohormones contain a 22-amino-acid signal peptide, one single copy of AKH, and a peptide portion termed adipokinetic hormone-associated peptide. The number and sequence of amino acid residues in the preprohormone of AKH III is surprisingly different from those of AKH I and II.

The processing of the preprohormones of AKH I and II has been elucidated in the closely related locust species *Schistocerca gregaria* (59); this species lacks AKH III (62). The signal peptide is co-translationally cleaved from the prepro-AKH, generating pro-AKH. Subsequent proteolytic processing is preceded by dimerization of two pro-AKHs (I/I, I/II, or II/II). Data on the precise processing and possible dimerization of pro-AKH III are lacking to date. In *L. migratoria*, the synthesis of the adipokinetic prohormones, their packaging into secretory granules, and their processing to the bioactive hormones is completed after approximately 75 min (63).

In situ hybridization showed that mRNA encoding *L. migratoria* prepro-AKHs are co-localized in the adipokinetic cells (14). Moreover, immuno-electron microscopic studies demonstrated that these three AKHs co-localize to the secretory granules (27; LF Harthoorn and DJ van der Horst, unpublished data). Consequently, in response to flight activity, the only known natural stimulus for the release of AKHs known, all three AKHs are released simultaneously.

Expression of the distinct AKH precursor genes is increased by flight activity. Northern blot analysis of the AKH precursor mRNAs in corpora cardiaca of locusts at rest and after a 1 h flight indicates that steady-state levels of AKH mRNAs are elevated; AKH I and II mRNAs increase approximately twofold, and AKH III mRNA increases approximately four times (14). There seems to be no acute need, however, for a marked increase in the production of AKH. Only a fraction of the total store of AKH is released during flight activity whereas AKHs are synthesized continuously, resulting in an increase in the amount of hormone stored in the adipokinetic cells with age (64), which is reflected by an increase in the number of secretory granules (28). On the other hand, Sharp-Baker et al (88, 89) discovered that, despite the huge stores of AKH, newly synthesised AKH molecules are preferentially released over older ones, suggesting that a major portion of the stored hormones belong to a non-releasable pool of older hormone. A coupling between the release of AKH and stimulation of hormone gene expression has recently been proposed at the level of signal transduction processes (61).

The adipokinetic cells in the corpus cardiacum appear to be subject to a multitude of regulatory, stimulating, inhibiting, and modulating substances as recently reviewed by Vullings et al (128) and Van der Horst et al (112). Neural influences come from secretomotor cells in the lateral part of the protocerebrum, via the nervus corporis cardiaci II. Up to now, only peptidergic factors have been established to be present in the neural fibers that make contact with the adipokinetic cells. Locustatachykinins initiate the release of AKHs (56, 57), whereas FMRFamide-related peptides inhibit the release of AKHs induced by release-initiating substances (127). Humoral factors that act on adipokinetic cells via the hemolymph are peptidergic and aminergic in nature. Crustacean cardioactive peptide initiates the release of AKHs (123), whereas the amines octopamine, dopamine, and serotonin only potentiate the effect of stimulation of the adipokinetic cells by release-initiating stimulatory substances (66). In addition, high concentrations of trehalose inhibit both the spontaneous release of AKHs and the release

induced by release-initiating substances (67). While these several substances may act in concert to regulate release of AKHs, their relative contributions during flight activity (or other conditions in which AKH may be implicated) remains to be established.

Activation and Specificity of Fat Body Lipases

In both vertebrates and invertebrates, sustained physical exercise is fueled largely by the oxidation of long chain fatty acids (FA), which are derived from stored TAG reserves. In vertebrates, FA are mobilized from TAG stores in adipose tissue, and a crucial role in this process is played by hormone-sensitive lipase (HSL). This enzyme controls the rate of lipolysis, catalyzing the first and rate-limiting step in the hydrolysis of the stored TAG, and also the subsequent hydrolysis of DAG and monoacylglycerols (MAG) (43). The FA liberated in adipose tissue are released into the blood and transported bound to serum albumin for uptake and oxidation in muscle.

Unlike FA in vertebrates, lipid in insects is released as DAG and transported to the flight muscles by lipophorin. At the flight muscles, DAG is hydrolyzed and the liberated FA is taken up and oxidized to provide energy. A clear functional similarity exists between vertebrate adipose tissue HSL and insect fat body TAG lipase, as both enzymes catalyze the hydrolysis of TAG stores to meet energy demands. However, there is an essential difference in the mode of action of both lipolytic enzymes. The vertebrate HSL catalyzes hydrolysis of TAG as well as DAG and MAG, resulting in the release of FA, whereas the action of the insect TAG lipase eventually causes the formation and release of DAG. How these differences in the mode of action are brought about is a physiologically relevant question that remains to be answered.

Regulation of Hormone-Sensitive Lipase in Vertebrate Adipocytes

HSL cDNA has been cloned from rat, human and mouse adipose tissue and the sequences obtained show a high degree of homology, whereas no significant relation to other mammalian lipases is discernable (60). Domain structure analyses suggest that HSL is composed of two domains: an N-terminal, presumably lipid-binding, domain and a C-terminal catalytic domain, containing the catalytic triad and a regulatory module containing potential phosphorylation sites. Specific residues have been identified in rat HSL as regulatory (Ser563) and basal (Ser565) sites. The regulatory site is phosphorylated in vitro by cAMP-dependent protein kinase (PKA), resulting in activation of HSL. AMP-activated protein kinase is the most likely kinase responsible for phosphorylation of the basal site in unstimulated adipocytes in vivo. Phosphorylation of the two sites seems to be mutually exclusive, suggesting that phosphorylation of the basal site has an antilipolytic role in vivo, and that phosphorylation of the regulatory site by PKA is preceded

by dephosphorylation of the basal site (43). Recently, two other PKA phosphorylation sites (Ser659 and Ser660) have been identified, and these novel sites were shown to be critical activity-controlling sites in rat HSL in vitro, while Ser563 plays a minor role in direct activation (1).

The mechanism behind activation of HSL upon phosphorylation by PKA is poorly understood. The mechanism seems to involve both translocation of HSL from the cytosol to the lipid droplet (30) and conformational changes in the HSL molecule. Different phosphorylation sites in HSL may play different roles in the process of translocation and increase the specific activity of the enzyme. In this connection it is worth noting that perilipins have been suggested to be involved in lipolysis (35, 36). Perilipins are a family of unique proteins intimately associated with the limiting surface of neutral lipid storage droplets. These proteins are acutely polyphosphorylated by PKA on lipolytic stimulation, hinting at a role in this process. Phosphorylated perilipin may serve as a docking protein for HSL, allowing lipase association only when cells are hormonally stimulated. Alternatively, conformational changes of phosphorylated perilipins may expose the neutral lipid cores of the lipid droplets, facilitating hydrolysis. Results of a recent study provide evidence in favor of the latter possibility, demonstrating that, in stimulated adipocytes, translocation of perilipin away from the lipid droplet occurs in concert with movement of HSL toward the droplet (17). Thus, perilipin might act as a barrier to deny HSL access to its lipid substrate in unstimulated adipocytes.

Regulation of Substrate Mobilization in Insects

Substrate mobilization for insect flight is controlled by AKHs, which are involved in both lipid and carbohydrate mobilization. Studies on signal transduction in flight-directed carbohydrate mobilization in *L. migratoria* have shown the involvement of G_s and G_q proteins (124, 126), cAMP and PKA (118, 120), inositol phosphates (121, 125) and Ca^{2+} ions (119) in AKH-induced activation of glycogen phosphorylase, the key enzyme in the conversion of fat body glycogen. Evidence was obtained for the presence of a store-operated Ca^{2+} entry mechanism in the fat body (122). In spite of the importance of lipid mobilization for sustained flight, our knowledge of the regulation of lipolysis in insect fat body is much more restricted, mainly due to technical problems in isolating or activating the lipase. The involvement of AKHs in lipolysis was demonstrated in vivo by an enhanced level of DAG in hemolymph of insects which had been injected with the hormones (reviewed in 10) and in vitro by the accumulation of DAG in isolated locust fat body tissue that was incubated with AKH (53, 134). In in vitro experiments, the effect of AKH on lipolysis was shown to be mediated at least in part by cAMP, suggesting a role for PKA in phosphorylation and activation of TAG lipase. Recently an approximate twofold increase in TAG lipase activity was reported in fat body of locusts injected with AKH-I (58).

In two insect species that rely on lipid mobilization during flight activity, it has been shown that the DAG in the hemolymph is stereospecific. While older data had demonstrated that the DAG released from the fat body of *L. migratoria* by the stimulatory action of AKH has the *sn*-1,2-configuration (52, 104), this finding was recently confirmed for *Manduca sexta* (5). The pathway for stereospecific synthesis of this *sn*-1,2-DAG is largely unknown. Proposed pathways involve stereospecific hydrolysis of TAG into *sn*-1,2-DAG by a stereospecific lipase acting at the *sn*-3 position of the TAG or hydrolysis of TAG into *sn*-2-MAG, followed by stereospecific reacylation of *sn*-2-MAG (reviewed in 111). A third suggested possibility is de novo synthesis of *sn*-1,2-DAG from *sn*-glycerol-3-phosphate via phosphatidic acid using FA produced by TAG hydrolysis (4).

Other experiments have provided more information about this important metabolic pathway. From young adult *M. sexta* fat body, a TAG lipase was purified and showed highest activity for TAG and DAG solubilized in Triton X-100 (4). Although the enzyme exhibited a preference for the primary ester bonds of acylglycerols, it did not show stereoselectivity toward either the *sn*-1 or *sn*-3 position of trioleoylglycerol. Phosphorylation of the enzyme by bovine heart PKA did not affect the activity of the enzyme toward TAG. It remains possible that, in the same manner as HSL (99), phosphorylation of fat body lipase in vivo results in translocation of the enzyme to the fat droplet, which may account for activation of the enzyme.

Because the main end products of TAG hydrolysis by the purified lipase were FA and *sn*-2-MAG and the lipase lacked stereospecificity, direct conversion of stored TAG to *sn*-1,2-DAG in *M. sexta* fat body stimulated by the action of AKH was concluded to be unlikely (4). However, the two alternative pathways for synthesis of *sn*-1,2-DAG appear to be unlikely as well. Studies on stereospecific acylation of *sn*-2-MAG catalyzed by a MAG-acyltransferase revealed that the activity of the transferase, which was found to be primarily a microsomal enzyme with only moderate stereospecificity, was not stimulated by AKH, nor did the size of the microsomal *sn*-2-MAG pool change during AKH-stimulated synthesis of *sn*-1,2-DAG (2). When decapitated adult *M. sexta* were used to measure lipid mobilization (3), AKH treatment showed that FA from TAG were converted to DAG without increasing the fat body content of either FA or phosphatidic acid, suggesting that the *sn*-glycerol-3-phosphate pathway is not involved either (5). The accumulation of *sn*-1,2-DAG led subsequent authors to conclude that the pathway for AKH-stimulated synthesis is stereospecific hydrolysis of TAG.

On the other hand, because this conclusion is in contrast with data obtained on both the stereospecificity and the end products of TAG hydrolysis by the purified fat body TAG lipase, one must speculate that this discrepancy could be an artifact of the experimental conditions under which the purified enzyme was tested in vitro (5). In addition, stereospecific hydrolysis of TAG into *sn*-1,2-DAG involves hydrolysis of the ester bond of the *sn*-3 fatty acid. The fate of this *sn*-3 fatty acid, which did not accumulate in the fat body or in the hemolymph, presently remains unclear.

Unlike the functional similarity between HSL in vertebrate adipose tissue and TAG lipase in insect fat body, a dicrepancy exists in the mode of action of both lipolytic enzymes. The vertebrate HSL catalyzes nonstereospecific hydrolysis of TAG as well as of DAG and MAG resulting in the release of FA, whereas the most likely stereospecific action of the insect TAG lipase causes the formation and release of *sn*-1,2-DAG. So far, the reason for this discrepancy is unknown, but both structural and regulatory aspects may be involved. Elucidation of the mechanism of TAG lipase action and the regulation of its activity is essential for a better insight into the poorly understood mechanism of flight-directed lipolysis in insects. In addition, data obtained may provide clues for a better understanding of the basic processes involved in activation and inactivation of HSL in vertebrates.

STRUCTURE OF LIPOPHORIN

Lipid transport via the circulatory system of animals constitutes a vital function that generally requires lipoprotein complexes, the apolipoprotein components of which serve to stabilize the lipids and modulate metabolism of the lipoprotein particle. For studies of plasma lipid transport, insects offer an attractive and relatively simple system. Because insects are physiologically highly active they require efficient lipid transport for a variety of specialised purposes. Hemolymph generally contains a single major lipoprotein particle, lipophorin, which is found in relatively large quantities. This multifunctional transport vehicle falls into the high-density lipoprotein class (high-density lipophorin, HDLp; d ~1.12 g/ml) and transports a variety of lipophilic biomolecules in the hemolymph. A characteristic feature of lipophorin is an ability to function as a reusable lipid shuttle by the selective loading and unloading of lipids at different target tissue sites (reviewed in 48, 72, 111). As a function of physiological (or developmental) needs for lipid distribution, lipophorin may exist in several forms with respect to relative lipid content and apolipoprotein composition, leading to differences in size and density of the particle. Thus, in species that rely on lipids during flight, lipophorins are loaded with additional DAG and exchangeable apolipoprotein, converting them to low-density lipophorin (LDLp), a particle with considerably more capacity to transport DAG fuel molecules between fat body and flight muscle. Lipophorin structures and that of flight-induced subspecies have been the subject of several recent reviews (11, 73, 74, 97, 113). Briefly, HDLp in insect hemolymph is a spherical particle in the range of $Mr = 450{,}000–600{,}000$. Its lipid cargo contains DAG as a major component, in addition to phospholipids, sterols, and hydrocarbons. In addition, lipophorin has been implicated in the transport of other hydrophobic ligands, such as juvenile hormone, pheromones, and carotenes, from internal biosynthetic sites to sites of utilization (37, 84, 87, 105, 106). Recent data on the lipid composition of HDLp of the yellow fever mosquito, *Aedes aegypti*, showed differences with other insect lipophorins studied to date.

In contrast to the usual prevalence of DAG, the most abundant neutral lipid in this lipophorin is TAG (32, 68).

The protein moiety of the HDLp particle is typically comprised of two integral, nontransferable glycosylated apolipoproteins, apolipophorin I (apoLp-I, ~240 kDa) and apolipophorin II (apoLp-II, ~80 kDa), which are invariably present in a 1:1 molar ratio. Recently, however, exceptions to this paradigm have been reported. Very recently, a different structure of HDLp apolipophorins was found in the cochineal scale insect, *Dactylopius confusus*: Although molecular mass and density of this lipophorin are in the normal range, two small glycosylated apolipophorins of approximately 25 and 22 kDa were isolated, the smaller one being the more abundant (149). Likewise, from the hemolymph of larval *Musca domestica*, in addition to HDLp, a second lipoprotein has been isolated which contains at least four small apolipoproteins of 20 to 26 kDa (25). It was suggested that the latter lipoprotein might have a role in pupal or adult cuticle formation.

Lipophorin Biosynthesis

In addition to its function in storage of lipids and carbohydrates, the insect fat body is the primary site of synthesis of proteins, including lipophorin. Depending on the species, lipophorin appears to be secreted into the hemolymph either as a nascent neutral lipid-deficient particle that loads specific lipids from other sites (reviewed in 49) or as a mature particle with a full complement of lipid (113). With respect to the TAG-rich *A. aegypti* lipophorin, wherein expression of apolipophorins is induced by a blood meal, the latter scenario appears to be the most plausible (115).

Studies of early events in lipophorin biosynthesis revealed that apoLp-I and -II arise from a common precursor (137). Using pulse-chase experiments and specific immunoprecipitation, a proapolipophorin was isolated from locust fat body homogenates, which was converted to apoLp-I and apoLp-II through post-translational cleavage. These results explain the 1:1 stoichiometry of these apolipoproteins in all lipophorin subspecies.

Important data on the cDNA and amino acid sequence of the apolipophorin precursor have recently become available for a few species. When a cDNA fragment (2.4 kb) coding for part of *L. migratoria* apoLp-I was challenged with fat body mRNA in a Northern hybridization, the length of the complete apolipophorin precursor was determined to be approximately 10.3 kb (113). A retinoid- and fatty acid-binding glycoprotein has been purified from *Drosophila melanogaster* heads, and its cDNA cloned (47). The 10.05 kb cDNA open reading frame encoded a 3351 amino acid protein that was identified as the *D. melanogaster* apolipophorin precursor. The N-terminal amino acid sequences determined for apoLp-I and -II of larval sheep blowfly *Lucilia cuprina* lipophorin (105), showed 54% and 70% identity to those of *D. melanogaster* apoLp-I and -II, respectively (47). At the same time, a 10.14 kb cDNA encoding the complete apolipophorin precursor of *M. sexta* was cloned and sequenced (100). The latter

two studies demonstrated that the precursor protein is arranged with apoLp-II at the N-terminus of proapolipophorin with apoLp-I at the C-terminus. In *D. melanogaster* proapolipophorin, a single consensus cleavage site, R*X*RR for dibasic endoprotease processing, was reported (47). It may be assumed that such a protease processes the precursor protein at this site, generating apoLp-II and apoLp-I. In another study, a cDNA fragment (1.2 kb) coding for part of *A. aegypti* apoLp-II was cloned and sequenced. Its deduced amino acid sequence showed approximately 40% identity with the corresponding regions of the *D. melanogaster* and *M. sexta* apoLp-II sequences (115). Northern blot analysis revealed that the size of *A. aegypti* apolipophorin precursor mRNA (approximately 10 kb) is similar to that of the other apolipophorin precursor mRNAs.

Recent studies, which included molecular characterization of the complete apolipophorin precursor cDNA from *L. migratoria* (J Bogerd & DJ van der Hosrt, unpublished data), revealed significant sequence homology among apolipophorin precursor proteins. The apolipophorin precursor protein of *L. migratoria* was 27% identical and 45% similar to that of *M. sexta* (100) and 25% identical and 43% similar to the retinoid- and fatty acid-binding glycoprotein of *D. melanogaster* (47). In addition, 35% identity and 52% similarity was observed with the partial, deduced apoLp-II amino acid sequence of *A. aegypti* (115). Moreover, statistically significant similarities were found between two distinct domains of locust apolipophorin precursor protein and two groups of extracellular proteins. The region between residues 31 and 1016 is similar to human apolipoprotein B-100 (21% identical and 38% similar; 46) and showed some similarity to various vertebrate and nematode vitellogenin precursors. Another region, located between residues 2816 and 3027, is related to the D domain of various von Willebrand factor precursors (23% to 26% identity, 39% to 42% similarity) and various mucins. The apolipophorin precursor proteins of *L. migratoria*, *D. melanogaster,* and *M. sexta* were used to identify contiguous conserved sequence motifs in alignments of large, nonexchangeable lipid transport proteins, demonstrating that the genes encoding the apolipophorin precursor—human apolipoprotein B, invertebrate and vertebrate vitellogenins, and the large subunit of the mammalian microsomal triaglyceride transfer protein—are members of the same multigene superfamily and are derived from a common ancestor gene (6).

Lipophorin Subspecies Interconversions

In insects capable of migratory flights, such as *L. migratoria* and *M. sexta*, AKH-induced mobilization of fat body TAG stores requires that this lipid be converted to DAG. DAG is released from the cell and loaded onto circulating HDLp particles, ultimately transforming them into LDLp ($d < 1.06$ g/ml). This loading process is facilitated by lipid transfer particles (see below) and requires that, concomitant with DAG uptake, several copies of the amphipathic exchangeable apolipoprotein, apolipophorin III (apoLp-III; ~18–20 kDa), associate with the

particle (140). Whereas HDLp is abundant during all developmental stages of the locust, apoLp-III expression is developmentally regulated and its hemolymph level is high only in adults (26). At the flight muscle, LDLp-associated DAG is hydrolyzed by a lipophorin lipase and the resulting free fatty acids are taken up and oxidized to provide energy. As the lipid content of the particle diminishes, apoLp-III dissociates with both constituents (HDLp and apoLp-III) recovered in the hemolymph. As such, they are free to return to the fat body for further DAG uptake, LDLp formation and transport. This process constitutes a vivid example of the lipophorin reusable shuttle hypothesis first proposed by Chino and coworkers.

APOLIPOPHORIN III

An important feature of all exchangeable apolipoproteins is an inherent structural adaptability, providing them with the ability to reversibly associate with circulating lipoproteins. It is implied, therefore, that these proteins can exist both in lipid-poor and lipid-associated states. Indeed, it is likely that exchangeable apolipoproteins can adopt more than one conformation in the lipid-bound state, as exemplified by human apolipoprotein A-I in disk structures versus spherical lipoproteins (31). Also, it is noteworthy that human apolipoprotein E (apoE) is not found on nascent chylomicron particles, yet acquisition of this apolipoprotein is essential for clearance of chylomicron remnant particles via receptor-mediated endocytosis (139). Importantly, the receptor recognition properties of apoE are manifest only upon lipid association, suggesting that a lipid-induced conformational adaptation may be a critical event in terms of this function. In another example, human apolipoprotein A-IV is known to partition between lipid-free and lipoprotein-associated states, perhaps serving as a reservoir of lipoprotein surface material (29).

Structural Properties

One of the best examples of the reversible existence of exchangeable apolipoproteins in lipid-free and lipoprotein-associated states is apoLp-III (74). In resting animals apoLp-III is recovered as a lipid-free hemolymph protein. However, during flight activity, or under the influence of AKH, apoLp-III associates with the surface of lipophorin, where it stabilizes DAG-enriched LDLp. Characterization of apoLp-III has revealed that it is rich in α-helical secondary structure. Sequence analysis reveals that the α-helices are amphipathic in nature, further suggesting a close similarity in structural properties with mammalian apolipoproteins (18, 93). Support for the concept that apoLp-III is structurally related to mammalian apolipoproteins also comes from comparison of the three-dimensional structures of *L. migratoria* apoLp-III and the 22 kDa N-terminal domain of human apoE (15, 144). These structures, which were determined for the proteins in the lipid-free state, reveal globular proteins comprised of a series of amphipathic α-helices,

organized in a bundle. ApoLp-III is a five-helix bundle while the N-terminal domain of human apoE is a four-helix bundle. In each case the helices are organized such that their hydrophobic faces orient toward the center of the bundle and their hydrophilic faces are directed at the aqueous media. This molecular architecture provides a rational explanation for the water solubility of these proteins in the absence of lipid and provides a framework for postulating conformational changes that may accompany lipid association.

Study of apoLp-III has been improved by the availablility of recombinant proteins (79, 95, 138). In the case of *L. migratoria* apoLp-III, in contrast to the natural protein (38), bacterially expressed protein is nonglycosylated. This feature allowed Soulages et al (95) to investigate the effect of apoLp-III carbohydrate on the lipid binding properties of this protein. Interestingly, these authors found that recombinant apoLp-III interacts with phospholipid vesicles more efficiently than natural apoLp-III and suggested that the carbohydrate structures on the protein may function to mask hydrophobic domains, which are important for lipid recognition. When it was discovered that *M. sexta* apoLp-III could be produced in bacteria at more than 100 mg/L culture (79), it became evident that this protein may be amenable to structure determination by heteronuclear multidimensional NMR techniques. Following confirmation that apoLp-III meets essential criteria for protein NMR including existence as a monomer at concentrations up to 1 mM and retention of a stable, native conformation for one week at 37° C in an appropriate buffer, a variety of three-dimensional NMR experiments were employed to obtain a complete assignment of apoLp-III NMR spectra (129). Recently, this information has been used to calculate a high-resolution solution structure (J Wang, BD Sykes, RO Ryan, unpublished data). This NMR structure, which represents the first solution structure determination of a full-length apolipoprotein, reveals a five-helix bundle architecture, which is similar to the X-ray crystal structures of *L. migratoria* apoLp-III and human apoE N-terminal domain. In addition, a short connecting helix was identified that links helix 3 and helix 4. It is noteworthy that a similar short helix is also present in human apoE N-terminal domain (144). We have proposed that this short helix may funtion in lipid recognition and/or initiation of binding to lipoprotein surfaces (129).

An important goal of apoLp-III NMR studies is to develop techniques that may be generally applicable to exchangeable apolipoproteins in their biologically active, lipid-associated state. Recently, a measure of success has been achieved in this endeavor through complex formation with the micelle-forming lipid, dodecylphosphocholine (131, 132, 138). Results obtained reveal significant spectral differences between lipid-free and lipid-bound conformations of apoLp-III, indicative of a major conformational change upon binding to lipid.

Open Conformation Model

We, and others, have obtained indirect experimental evidence in support of the conformational opening hypothesis. Early studies, using a monolayer balance, revealed that apoLp-III occupies more area at the air/water interface than can be

explained if the globular state was retained (44). In studies of the properties of apoLp-III in association with model phospholipids, Wientzek et al (143) and Weers et al (135) showed it is capable of transforming bilayer vesicles of dimyristoylphosphatidylcholine (DMPC) into uniform disk-like particles. Characterization of these complexes revealed that five or six apoLp-III molecules associate with each disk and, based on compositional analysis and the molecular dimensions of the protein, it was hypothesized that apoLp-III adopts an open conformation and aligns around the perimeter of the bilayer structure with its α-helices oriented perpendicular to the fatty acyl chains of the phospholipids (74, 143). Subsequently, spectroscopic evidence in support of this interpretation has been reported (69). In another study, Raussens et al (70) found that association with lipid has a significant effect on the amide proton hydrogen/deuterium exchange rate in apoLp-III, further suggesting that lipid association induces a change in protein structure that alters the solvent accessibility of amino acid residues in the protein.

Using an alternate approach to investigate conformational changes associated with lipid interaction, site-directed mutagenesis has been employed to introduce two cysteine residues into apoLp-III, which were designed to permit formation of a disulfide bond (54). Characterization of the mutant protein revealed that it contained the desired mutations and that it folds properly in solution. Furthermore, under oxidizing conditions, the cysteine residues (which are located in the loop segments that connect helices 1 and 2 and 3 and 4) spontaneously form a disulfide bond. Subsequent study of reduced and oxidized mutant apoLp-III revealed that formation of the disulfide bond abolishes the ability of the protein to interact with lipoprotein surfaces. Upon reduction of the disulfide bond, however, full lipoprotein binding activity is restored. The data suggests that disulfide bond formation tethers the helix bundle, preventing exposure of its hydrophobic interior through conformational opening. As such, these data support the hypothesis that apoLp-III opens about ''hinged'' loop regions to create an elongated amphipathic structure that represents the lipid binding conformation of the protein.

Lipid Binding Properties

Early studies (140) showed a correlation between the DAG content of lipophorins and the amount of apoLp-III associated. Since that time several strategies have been employed to characterize this relationship. Enrichment of isolated lipoproteins with DAG via facilitated lipid transfer (92) or direct addition of exogenous short chain di C_8 DAG (98) both induce apoLp-III association with lipoprotein surfaces. In another approach, treatment of isolated lipoproteins with phospholipase C to create DAG (51, 90) causes exchangeable apolipoprotein association as a function of the extent of conversion of surface phospholipid into DAG. Taken together, these data support a model wherein apoLp-III binding to the surface monolayer of lipoproteins involves an interaction between the amphipathic α-helices of the protein with phospholipid and DAG (74, 130). It is reasonable to

consider that positively charged amino acids, located at the edge of the helices, form salt bridges either with adjacent helices or with charged groups on the lipid (148).

In terms of the initiation of lipoprotein association, studies have been performed using apoLp-III as a model system. It has been suggested that electrostatic interactions between amino acid side chains and phospholipids may localize the apoprotein at the particle surface (148). In cases where the lipoprotein has available hydrophobic binding sites or surface defects, a conformational change ensues and a stable binding interaction occurs. This process can be reversed by metabolic events that reduce the available hydrophobic surface, wherein the apolipoprotein would be released from the particle and adopt its solution conformation.

An alternate hypothesis (93, 96) proposes that apolipoprotein binding is regulated by a small region of hydrophobic amino acids, a ''hydrophobic sensor,'' located at one end of the apolipoprotein. These workers noted that apoLp-III molecules from several insect species contain a region of exposed hydrophobic amino acids in the loop regions connecting helices 1 and 2 and helices 3 and 4. It has been proposed that these amino acids play a critical role in initiation of binding, associating with the lipoprotein with the long axis of the protein perpendicular to the particle surface. In the presence of sufficient surface-exposed hydrophobic material, the protein opens to form a stable interaction (54). Evidence in favor of the ''hydrophobic sensor'' mechanism has been obtained through experiments employing surface plasmon resonance spectroscopy (96), and indirectly from studies with disulfide crosslinked apoLp-III (54). More recently, Soulages & Bendavid (94) have extended this model, providing evidence that *M. sexta* apoLp-III interaction with phospholipid vesicles is greatly affected by solution pH. These authors propose that below pH 6.5, apoLp-III adopts a partially folded ''molten globule'' conformation with an increased affinity for binding to phosphatidylcholine vesicles. This hypothesis has been examined further in both *M. sexta* and *L. migratoria* apoLp-III (55, 136). In both studies, site-directed mutagenesis was employed to replace specific residues in apoLp-III and the effect of such changes on lipid binding evaluated. In *M. sexta* apoLp-III, it was demonstrated that valine 97, located in the approximate center of the short connecting helix (129) plays a critical role in the lipoprotein binding ability of the protein (55). In the case of *L. migratoria* apoLp-III, Weers et al (136) mutagenized key leucine residues in the loops connecting helices 1 and 2 and helices 3 and 4 (93). Upon conversion of these residues to arginine it was noted that, although the lipoprotein binding ability of the protein was decreased, the mutant protein displayed an increased ability to disrupt phospholipid bilayer vesicles. On the basis of these results, it was suggested that electrostatic attraction plays an important role in phospholipid binding while hydrophobic interactions are an important force in lipoprotein binding. Although both mutagenesis studies suggest apoLp-III interacts with lipid surfaces specifically via one of its ends, the precise mechanism of reversible lipid binding remains to be determined.

LIPID TRANSFER PARTICLE

Since the discovery by Zilversmit et al (150) of specialized proteins that function in the redistribution of hydrophobic lipid molecules, a great deal of interest in this class of protein has been generated. A wide variety of distinct lipid transfer proteins have been characterized and their metabolic roles investigated. Among these are the human plasma cholesteryl ester and phospholipid transfer proteins (103), microsomal triglyceride transfer protein (MTP; 141) and the intracelleular phospholipid transfer proteins (145).

The lipophorin shuttle hypothesis, with repeated cycles of lipid loading and depletion, implies the existence of additional factors to facilitate these interconversions. A heat-labile factor (78, 81), now termed lipid transfer particle (LTP), was isolated in 1986 and shown to facilitate vectorial redistribution of lipids among plasma lipophorin subspecies. In addition, a flight muscle lipase has been described which is hypothesized to lipolyze lipophorin-associated DAG at the flight muscle tissue (116, 142). In subsequent studies LTP was strongly implicated to function in vivo in formation of LDLp from HDLp in response to adipokinetic hormone (114) as well as in the conversion of HDLp into egg very high density lipophorin upon receptor mediated endocytosis into oocytes (50). The apparent obligatory role of LTP in hemolymph lipoprotein conversions is analagous to the recent postulate that mammalian MTP plays an important role in the biogenesis of triacylglycerol-rich lipoproteins (141). The concept that LTP functions in flight-related lipophorin conversions is congruent with an increased hemolymph concentration of LTP in adults versus other developmental stages (106, 117).

Physical and Structural Properties

Compared to other types of lipid transfer catalyst, *M. sexta* LTP exhibits novel structural characteristics. Data from early studies indicate it is a high molecular weight complex of three apoproteins (apoLTP-I, Mr ~320,000; apoLTP-II, Mr = 85,000 and apoLTP-III, Mr = 55,000) and 14% noncovalently associated lipid (80). Interestingly, catalysts with similar structural properties have been isolated from *Locusta migratoria*, *Periplaneta americana* and *Bombyx mori* hemolymph (42, 102, 107). The lipid component resembles that of lipophorin in that it contains predominantly phospholipid and DAG (72). Its apparent large size, together with the presence of lipid, have been employed in the purification scheme to isolate LTP (81). An important question arising from these physical characteristics relates to the requirement of the lipid component as a structural entity and/or its involvement in catalysis of lipid transfer. Although its precise function is not known, studies employing lipoproteins containing radiolabeled lipids in incubations with LTP revealed that the lipid component of the particle is in dynamic equilibrium with that of lipoprotein substrates (80). Thus, it was concluded that the lipid moiety of LTP is not merely a static structural entity of the particle, but instead may play a key role in facilitation of lipid transfer.

The morphology of LTP has been investigated by electron microscopy (77, 102). LTP is a highly asymmetric structure with two major structural features: a roughly spherical head and an elongated, cylindrical tail, which has a central hinge. Sedimentation equilibrium experiments conducted in the analytical ultracentrifuge revealed a native particle molecular weight of approximately 900,000 (76). This data agrees with results obtained from native pore-limiting gradient gel electrophoresis (80) and calculations based on the dimensions of LTP determined by electron microscopy. In sedimentation velocity experiments evidence of a reversible, ionic-strength–dependent aggregation of LTP was obtained. Both aggregated and monomeric LTP are catalytically active, however, and have similar amounts of secondary structure conformers, indicating that large changes in structure do not accompany ionic strength-induced changes in sedimentation coefficient. The ability of LTP to self associate may be important in its physiological function because ionic strength conditions in hemolymph would favor formation of aggregated LTP (76). The presence of a 150-fold excess of lipophorin in hemolymph, however, may dictate that LTP interact with these potential substrates rather than self associate, thereby facilitating its biological function. It is possible that a differential affinity of LTP for lipid particle substrates or biomembranes, on the basis of their lipid content, net charge, or otherwise, may be a factor in modulating activity of LTP.

Catalytic Activity and Substrate Specificity

A number of studies have investigated the question of whether LTP is capable of facilitating lipid transfer to or from substrate lipid particles other than lipophorins. Ryan et al (75) showed that LTP can facilitate lipid transfer between lipophorin and an unrelated hemolymph chromolipoprotein from *Heliothis zea*. The observation that LTP can mediate DAG exchange between these substrate particles suggests it may function in vivo to redistribute lipid among lipophorin and unrelated very-high-density lipoproteins present in hemolymph.

In related experiments examining the ability of LTP to utilize other substrates, the ability to transfer lipid between lipophorin and human LDL has been examined (82). When lipophorin and LDL were incubated together in the absence of LTP and re-isolated by density gradient ultracentrifugation, no changes in their respective density distribution were detected. When catalytic amounts of LTP were added, however, a dramatic shift in the lipoprotein density profile was seen with LDL floating to a lower-density position and lipophorin recovered at a higher-density position. Neither apoprotein transfer nor particle fusion were responsible for these changes in lipoprotein density. When the lipophorin substrate was labeled with [^{3}H]DAG, it was shown that LTP-mediated vectorial net transfer of DAG from lipophorin to LDL was responsible for the observed changes in lipoprotein density profile. These data provide strong support for the concept that transfer activity inherent in LTP is sufficent to induce major changes in lipophorin lipid content and composition that normally occur in vivo. Further-

more, the data suggest that the direction and extent of LTP-mediated lipid redistribution is dependent upon the structural properties and hydrophobic environment provided by potential lipid donors and acceptors.

In other studies of the lipid substrate specificity of LTP, [1-^{14}C]acetate has been used to label the DAG and hydrocarbon moiety of lipophorin in vivo (91). Subsequent transfer experiments revealed that LTP is capable of facilitating transfer of hydrocarbon from lipophorin to LDL, suggesting LTP may play a role in movement of these extremely hydrophobic, specialized lipids from their site of synthesis to their site of deposition at the cuticle (see also 102). When acceptor LDL particles were analyzed prior to complete transfer of lipophorin-associated lipid it was observed that DAG was transferred preferentially during the initial stage of the reaction after which hydrocarbon transfer increased. Recently, *B. mori* LTP was demonstrated to facilitate transfer of carotenes among lipophorin particles (106). Again, when compared to DAG transfer, the rate of LTP-mediated carotene redistribution was much slower. Taken together, these results suggest that LTP may have a preference for DAG rather than hydrocarbon or carotenes. Alternatively, the observed preference for DAG may be a function of the relative accessibility of the substrates within the donor lipoprotein.

Mechanism of LTP-Mediated Lipid Transfer

A major question regarding the ability of LTP to facilitate net vectorial lipid transfer relates to the mechanism of this process. Transfer catalysts may act as carriers of lipid between donor and acceptor lipoproteins or, alternatively, transfer may require formation of a ternary complex between donor, acceptor and LTP. Based on the well characterized LTP-mediated vectorial transfer of DAG from HDLp to human LDL (82) a strategy was developed to address this question experimentally (13). It had been previously shown that, in solution, LTP catalyzes an extensive vectorial net transfer of DAG from lipophorin to LDL. LDL particles become enriched in DAG while lipophorin particles are depleted of neutral core lipid (82). This reaction is not accompanied by apoprotein exchange or transfer and DAG is not metabolized during this reaction. [^{3}H]DAG-HDLp and unlabeled LDL were covalently bound to Sepharose matrices and packed into separate columns connected in series, whereafter LTP or buffer was circulated. Control experiments revealed that the lipoproteins remained fixed to the solid phase and that DAG did not transfer to LDL in the absence of LTP. In the presence of LTP, however, there was a concentration-dependent increase in the amount of labeled DAG recovered in the LDL fraction, demonstrating that LTP can facilitate net lipid transfer via a carrier-mediated mechanism.

In another approach, Blacklock & Ryan (12) employed apolipoprotein-specific antibodies to probe the structure and catalytic properties of *M. sexta* LTP. In antibody inhibition studies, evidence was obtained that apoLTP-II is a catalytically important apoprotein. In a separate study, Van Heusden and coworkers (117) provided evidence from antibody inhibition studies that all three LTP apoproteins

are important for lipid transfer activity. Furthermore, evidence was obtained that, unlike apoLp-III, apoLTP-III is not found as a free protein in hemolymph (117) although it can dissociate from the complex upon treatment with a nonionic detergent, while apoLTP-I and -II remain associated (12).

LIPOPHORIN RECEPTORS

A major difference between insect lipophorins and vertebrate lipoproteins is the selective mechanism by which insect lipoproteins transport their hydrophobic cargo. For example, at the fat body cell, DAG can be loaded onto circulating HDLp particles for lipid mobilization during flight activity, an adult-specific process. DAG may also be extracted from HDLp for lipid storage, a prominent process in larval and younger adult insects. It is likely that for both lipid removal and accretion, interaction between lipophorins and fat body cells takes place. In *L. migratoria*, a high-affinity HDLp binding site ($Kd \sim 10^{-7}$ M) has been characterized in intact fat body tissue as well as in fat body membranes of larval and adult locusts (23, 86, 110). The interaction between HDLp and this binding site did not require Ca^{2+}-ions (23), in contrast to other insect lipoprotein receptors (71, 109, 133). The number of HDLp binding sites at the locust fat body cell surface increased between day seven and day 11 after imaginal ecdysis (23), suggesting a role in an adult-specific process such as flight activity—this increase coincides with changes in flight muscle metabolism required for flight activity (9, 39). In adult fat body tissue, no differences were observed in the binding characteristics of AKH-injected and control locusts (23), suggesting that if this HDLp binding site is involved in LDLp formation, the abundance of these sites is sufficient to cope with the increased lipid mobilization during sustained fligh activity. As yet, however, no conclusive evidence has been presented for the involvement of HDLp binding sites in LDLp formation, although both HDLp binding and LDLp formation are inhibited by monoclonal antibodies specific for apoLp-II and not by those directed against apoLp-I (41, 86).

Interaction of Lipophorin with Fat Body Cells

Recently, the HDLp binding site in larval and young adult locusts was identified as an endocytic receptor involved in uptake of HDLp (19). Endocytic internalization of HDLp appeared to be developmentally down-regulated in the adult stage. When insects were starved, however, internalization of HDLp remained evident. Also, in larvae of the dragon fly, *Aeshna cyanea*, it had been shown that HDLp is internalized by the fat body (7), although this was not observed in the midgut epithelium (8), implying that endocytosis of HDLp does not occur in all target tissues. The specific HDLp binding sites identified in fat body and gut of larval *M. sexta* appear not to be involved in endocytotic uptake of this ligand (108, 109). Although endocytic uptake of HDLp seems to conflict with the selec-

tive process of lipid transport between HDLp and fat body cells without degradation of the lipophorin matrix, the pathway followed by the internalized HDLp may be different from the classical endosomal/lysosomal pathway. In studies with radiolabelled HDLp neither substantial degradation nor accumulation of HDLp in fat body cells were apparent (19). On the basis of these results it was proposed that lipid transport may occur by a retroendocytic pathway, as previously postulated for larval dragon fly fat body by Baurfeind & Komnick (7). Internalization of HDLp by locust fat body cells has been studied at the electron-microscopic level using ultrasmall gold-labeled HDLp and fluorescent dye (DiI)-labeled HDLp (20). In the latter experiments, visualization of the DiI-labeled lipophorin was achieved by diaminobenzidine photoconversion (21). Internalized labeled HDLp was observed in the endosomal/lysosomal compartment of fat body cells of both young and older adults, although labeling in older adults was much less abundant. Still, in view of the accumulation of HDLp in lysosomes, whether part of the internalized HDLp is re-secreted after intracellular trafficking and possible unloading of lipid cargo must be evaluated. The function of receptor-mediated endocytosis remains unclear because inhibition of endocytosis did not reduce the exchange of DAG or cholesterol between HDLp and the fat body cell (19).

Human Low-Density Lipoprotein Receptor Homologue

Very recently, the putuative receptor involved in endocytic uptake of HDLp in the locust fat body has been identified (22). Receptor-mediated endocytosis of lipoproteins has been studied in detail in vertebrate cells (34). Cloning of different receptors that function in lipoprotein uptake has resulted in the identification of the LDL receptor family, the members of which share structural and functional features (16, 24, 40, 45, 101, 146). These receptors appear to originate from an ancient receptor in view of the identification of a similarly composed cell surface molecule in *Caenorhabditis elegans* (147) and two insect vitellogenin receptors belonging to this gene superfamily (83, 85). The endocytic uptake of HDLp by locust fat body cells was therefore hypothesized to be mediated by a member of the LDL receptor family (22). A novel member of the LDL receptor family was cloned and sequenced. Northern blot analysis revealed expression in locust fat body as well as in oocytes, brain, and midgut. This receptor appeared to be a homologue of the mammalian very-low-density lipoprotein receptor: it contains eight cysteine-rich repeats in its putative ligand-binding domain. This receptor represents the first identification of an invertebrate LDL receptor family member with an extracellular domain composed of a single ligand-binding domain and EGF-precursor domain, a receptor organization that has been found in many vertebrates. When expressed in COS-7 cells, the receptor mediates endocytic uptake of HDLp. Expression of the receptor mRNA in fat body cells is down-regulated during adult development, which is consistent with the previously reported down-regulation of receptor-mediated endocytosis of lipophorins in fat body tissue (19).

DIRECTIONS FOR FUTURE RESEARCH

In assessing the current status of this research field, we note that studies of the regulation of insect lipid mobilization and transport during flight activity has led to a valuable research model. Knowledge of the key events of this process have provided a coherent understanding as well as an integrated view. In terms of future exploration, the multifactorial process of AKH release represents an important ongoing research effort, whereas neuropeptide-receptor binding interactions will require considerable research effort. The mechanisms whereby neuropeptides regulate and modulate a sequence of metabolic processes, including the mobilization of DAG through activation of fat body TAG lipase is another important future research goal. Transport of lipid released in the hemolymph requires the hormone-stimulated transformation of lipophorins, which are capable of alternating between a relatively lipid-poor and lipid-enriched forms. In these reversible conversions the exchangeable apolipoprotein, apoLp-III, which exists in alternate lipid-free and a lipid-bound states, plays an essential role. On all aspects of lipophorin (structure, biosynthesis, interconversion of subspecies and evolutionary relationships) considerable information has been gained, but much additional data is required for a full understanding of this unique system and to fully apply insight into corresponding processes in higher organisms.

The lack of sequence information on apoprotein components is an impediment to a fuller understanding of LTP structure and activity. This information is critical to addressing questions regarding the specific roles played by various subunits of the holoparticle. Since the discovery of LTP, considerable information has been gained about its in vivo function, lipid and lipid particle substrate specificity, mechanism of action, and morphology. In the future it will be important to extend this knowledge by employing molecular biology techniques to clone and sequence LTP apoproteins and to further investigate the structure and interactions of this unique catalyst. Important progress in the area of insect lipoprotein receptors has opened the door to a potential understanding of the molecular basis of the lipophorin shuttle hypothesis and its integration with emerging endocytic pathways for lipoprotein uptake from hemolymph. Through continued exploitation of the remarkable physiology surrounding flight related lipid transport, this model system will continue to provide fundamental discoveries of profound biological significance.

ACKNOWLEDGMENTS

This project was supported by a NATO collaborative research grant to DJ Van der Horst and RO Ryan. Research support from the Alberta Heritage Foundation for Medical Research and the Medical Research Council of Canada to RO Ryan is gratefully acknowledged. Support was provided by a grant from the Life Sci-

ence Foundation (SLW), subsidized by the Netherlands Organization for Scientific Research (NWO).

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Volume 45, 2000

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