Insect Immune Activation by Apolipophorin III Is Correlated with the Lipid-Binding Properties of This Protein†

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ABSTRACT: Apolipophorin III (apoLp-III) is an exchangeable insect apolipoprotein consisting of five amphipathic α-helices. The protein is able to open reversibly on associating with hydrophobic surfaces and plays a role both in lipid transport and induction of immune responses. Point mutations were introduced at positions 66 (N→D) and/or 68 (K→E) between helices 2 and 3, a region possibly serving as a hinge for the opening of the molecule when associating with lipids. The lipid-binding properties of the mutant proteins were analyzed and compared with their immune inducing activities. Structural properties of the proteins were studied by far UV circular dichroism spectroscopy and their abilities to form discoidal complexes of dimyristoyl phosphatidylcholine (DMPC) vesicles were investigated. In comparison to wild-type apoLp-III, apoLp-III(N66D/K68E), and apoLp-III(K68E) displayed significantly decreased lipid-binding abilities and immune stimulating activities, while these effects were less noticeable with apoLp-III(N66D). The secondary structure of the double mutant apoLp-III(N66D/K68E) was similar to that of wild-type apoLp-III. A noticeable reduction of α-helical content could be observed for the single mutants apoLp-III(N66D) and apoLp-III(K68E), which was accompanied by an increase in percentage amount of *β*-turns. The stability of the secondary structure determined by heat denaturation was not affected by mutagenesis. Furthermore, the ability of all proteins to form discoidal complexes of equal size and shape in the presence of dimyristoyl phosphatidylcholine indicated that the mutagenesis did not affect the molecular architecture in the lipid-associated conformation. The relationship between reduced lipid association and reduced immune stimulating activity supports the hypothesis that apoLp-III-induced immune activation is triggered by the conformational change of the protein.

Apolipophorin III (apoLp-III)[1] is an abundant hemolymph protein of insects from several orders. It is a globular exchangeable apolipoprotein of approximately 18 kDa in mass with a high α-helical content in secondary structure. A common feature of apoLp-III and other exchangeable proteins such as human apolipoprotein (apo) E, apo A1 or apo C is the ability to reversibly bind to hydrophobic surfaces of lipoproteins.

The role of apoLp-III for the lipid metablism of adult insects is well understood and summarized in the lipid shuttle hypothesis (reviewed in ref *1*). During phases of high energy turnover, diacylglycerol (DAG) is mobilized from the fat body and high-density lipophorins (HDLp) are loaded with DAG. This lipid loading is accompanied by swelling of the lipoprotein particle leading to successive reduction of its buoyant density and presentation of DAG at its surface. Meanwhile apoLp-III-molecules associate with the lipophorin particle converting it to a stable low-density lipophorin (LDLp), which serves as transport vehicle for lipids in hemolymph. At the target tissue, DAG is deloaded from LDLp, while apoLp-III dissociates from the lipoprotein. Both apoLp-III and lipophorin can be reused for another cycle of DAG transport. Lipid association and dissociation of apoLp-III is accompanied by a conformational change from a protein globule in the lipid free state to an unfolded conformation when bound to lipids.

The majority of research to date focuses on the structural and conformational changes to understand the molecular mechanism of protein unfolding in LDLp-biogenesis (reviewed in ref *2*). The molecular architecture of apoLp-III from two different insect species, *Locusta migratoria* and *Manduca sexta*, could be established by X-ray crystallography (*3*) or by three-dimensional heteronuclear NMR spectroscopy, respectively (*4*). Both structures suggest a similar arrangement of five elongated amphipathic α-helices (helices 1−5) connected by short loops. In contrast to *L.*

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[1] Abbreviations: Apo, apolipoprotein; apoLp-III, apolipophorin III; CD, circular dichroism; DAG, diacylglycerol; DMPC, dimyristoylphosphatidylcholine; FPLC, fast-protein liquid chromatography; HDLp, high-density lipophorin; LDL, low-density lipoprotein; LDLp, low-density lipophorin; MALDI-TOF, matrix-assisted laser desorption ionization−time-of-flight; PL-C, phospholipase C; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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migratoria-apoLp-III, however, the *M. sexta* protein is nonglycosylated, remarkably different in primary structure and contains an additional short minihelix (3′) between helices 3 and 4. In the globular, water soluble state the hydrophobic residues of the helices are orientated toward the proteins' interior. Upon lipid binding, the conformational change of apoLp-III is supposed to function as a reversible opening, directing the hydrophobic residues of the helices against the hydrophobic surface. The molecular mechanisms of this opening are not understood in detail, and currently, two models of lipid binding are discussed. The first one derives from the structural analysis of *L. migratoria*-apoLp-III (*3*). Here, conserved hydrophobic residues in the loop regions between helices 1 and 2 and between helices 3 and 4 orientate the protein to lipid surfaces. After lipid recognition, opening of the molecule occurs by moving helices 3 and 4 away from helices 1, 2, and 5 using the loop regions between helices 2 and 3 and helices 4 and 5 as hinges. This model has been confirmed by studies on *M. sexta*-apoLp-III, where a valine residue at position 97 within the minihelix-3′ turned out to play a key role in initiating lipid binding (*5*). Moreover, mutagenesis of the *Locusta* protein showed that hydrophobic residues between helices 1 and 2 or helices 3 and 4 act as putative "sensor" loops for lipid binding (*6*). The second model of lipid binding results from fluorescence anisotropy spectroscopy and near UV circular dichroism (CD) spectroscopy studies performed with *L. migratoria* apoLp-III (*7*). It suggests that opening of apoLp-III results from a separation of helix-1 from helix-5, whose interaction in the globular conformation might be caused by weak hydrogen bonding.

This progress, however, has so far not led to sufficient explanation for the existence of apoLp-III in larval hemolymph. The lipid shuttle hypothesis is valid only to adult insects in phases of high catabolic activity. The larval lipid metabolism is focused on storage of lipids, and LDLp particles are not detectable in larval hemolymph (*8*). Therefore, the possibility of additional physiological relevances of apoLp-III is discussed in recent reports. For example, in metamorphosis, upregulation of apoLp-III-mRNA has been detected during programmed cell death of intersegmental muscle cells and neurons (*9*). Iimura et al. (*10*) reported a role of apoLp-III in hemagglutination, and Halwani and Dunphy suggested that the protein might have a synergistic effect with insect lysozyme (*11*).

Previous work in our group demonstrated that the intrahemocoelic injection of purified *Galleria mellonella*-apoLp-III as well as of the recombinant protein into untreated larvae leads to a linear dose-dependent increase in hemolymph titers of antimicrobially active molecules (*12*). As both immune stimulation by microbial provocators and by apoLp-III is not correlated with a remarkable increase in hemolymph concentration of apoLp-III (*12*) or increase in endogenous apoLp-III expression (*13*), the immune activating capability of apoLp-III must reside in the features of the protein itself. A recent study, which investigated the biophysical and lipid-binding properties of a C-terminal truncated mutant of apoLp-III, proved that the remaining N-terminal fragment comprising helices 1−3 retained its ability to bind to lipids (*14*). This fragment is also able to trigger immune responses (Dettloff, unpublished results), suggesting the possibility of a relationship between immune-inducing activity and lipid-binding ability of apoLp-III. Therefore, focusing our interest on the first three N-terminal helices, we subjected the wild-type protein to site-directed mutagenesis, which affected the putative hinge (*15*) between helices 2 and 3. Consequences of point mutations within this hinge region have so far not been been reported. Amino acid exchanges at positions 66 and/or 68 leading to a change in net charges at the indicated positions were performed, and structural, lipid-binding, and immune activating properties of the mutant proteins were investigated. The results support the idea of a relationship between the lipid-binding ability and the immune modulatory property of apoLp-III and indicate that immune activation by apoLp-III is triggered by a conformational change.

EXPERIMENTAL PROCEDURES

Rearing of G. mellonella Larvae. *G. mellonella* larvae were reared as described previously (*16*). Larvae weighing between 280 and 330 mg were used throughout this study.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using PCR with mismatched primers. A 5′-cDNA fragment comprising the nucleotide sequence 148−345 and a 3′-cDNA fragment comprising the nucleotide sequence 346−492 was amplified from a plasmid harboring the full-length cDNA template (*13*) by pfu-polymerase (Stratagene). Using the primer pairs 5′-CCGACGCGTCCACGCCGCTGCA and 5′-GTCCGCGTCGTTCAGCGCTTTCTG, and the primer pair 5′-GGCGAGGCGAAGGAGGCGCTGGAG and 5′-GCCGGAATTCTTACTGCTTGCTGGCGGCCTC, respectively, mismatched nucleotides were introduced in two cDNA fragments at nucleotide positions 343 and 349. Amplification of two other cDNA fragments comprising the residual nucleotide sequences missing in the mismatched cDNA fragments above was performed using the primer pairs 5′-CCGACGCGTCCACGCCGCTGCA, 5′-GTTCGCGTCGTTCAGCGCTTT, and 5′-GGCAAGGCGAAGGAGGCGCTG, 5′-GCCGGAATTCTTACTGCTTGCTGGCGGCCTC, respectively. The two longer PCR products were phosphorylated (Promega) by 10 units of T4 polynucleotide kinase (New England Biolabs) in the presence of 1 mM ATP. Full-length cDNAs with the desired mismatches at the indicated positions were generated after PCR amplification from ligation reactions containing one or both mismatched cDNA fragments using the primer pair 5′-CCGACGCGTCCACGCCGCTGCA and 5′-GCCGGAATTCTTACTGCTTGCTGGCGGCCTC. Following full-length cDNA amplification, PCR products were ligated into the pET 22b+ vector (Novagen) as described by Ryan et al. (*17*). The plasmids were transfected into *Escherichia coli* BL 21 (DE3) cells. DNA sequences of the vector constructs were verified by cycle sequencing using SequiTherm EXCEL II DNA Sequencing Kit-LC (Biozym) and an automated DNA sequencer (LICOR 4000 L, MWG-Biotech).

Expression and Purification of the Recombinant Proteins. Expression of recombinant apoLp-III was performed as described previously (*13*). After 4 h of expression in M9 minimal salts (*18*) at 37 °C, cells were removed by centrifugation (20 min, 6 500 rpm, Beckman JA10), the supernatants containing the recombinant proteins were filtrated (0.2 μm) and concentrated by 10 kDa ultrafiltration (Amicon). Concentrated supernatants were exchanged against 20 mM BisTris [pH 6.5 for apoLp-III; pH 6.2 for apoLp-

III(N66D), and pH 5.9 for apoLp-III(K68E) and apoLp-III-(N66D/K68E, respectively]. Protein solutions were applied to a 20 mL-DEAE-Sepharose CL 6B anion-exchange column (Sigma) equilibrated with the equivalent buffers. Wild-type apoLp-III and apoLp-III(N66D) were detected in the late fractions of the flow-through, while apoLp-III(K68E) and apoLp-III(N66D/K68E) were eluted with a gradient from 0 to 100 mM NaCl. After ion-exchange chromatography, proteins were exchanged against PBS and further purified by gel filtration on a HiLoad 16/60-Superdex column using FPLC (Pharmacia). Following gel filtration, proteins were exchanged against sterile distilled water by ultrafiltration, lyophilized, and stored at −20 °C. Protein purity was controlled by SDS−PAGE, following staining with Coomassie Brilliant blue R 250 according to Laemmli (*19*), and by MALDI-TOF mass spectrometry analysis.

Protein Determination. Determination of protein concentrations was performed using the BCA-assay (Pierce) with bovine serum albumin (BSA) as standard. For UV CD spectroscopy, after protein hydrolysis in 6 M HCl/7% (w/v) thioglycolacid for 24 h at 110 °C, protein content was calculated by amino acid analysis using an automated amino acid analyzer (Sykam).

MALDI-TOF Mass Spectrometry Analysis. A Bruker Reflex MALDI mass spectrometer equipped with delayed extraction was used with α-cyano-4-hydroxycinnamic acid as a matrix.

UV CD Spectroscopy. Circular dichroism is observed by different left- and right-handed circular polarized light absorbance of optically active molecules. It was used to analyze the secondary structure of apoLp-III and the mutant proteins and to follow the changes in folding as a function of temperature. UV circular dichroism spectroscopy was carried out using a Jasco J 600 spectropolarimeter. Proteins were dissolved in 100 mM sodium phosphate (pH 7.5) at a concentration of 100 μg/mL. CD spectra were evaluated with the help of a modified Contin program by Provencher and Glöckner (*20*), which uses poly-L-glutamate as α-helical reference standard.

Preparation of apoLp-III-DMPC-Complexes. ApoLp-III-dimyristoyl phosphatidylcholine (DMPC) complexes were prepared as described by Wientzek et al. (*21*). First, 12.5 mg of DMPC (Bachem) were dissolved in 500 μL of chloroform/methanol (3:1). The organic solvent was evaporated to dryness by exposure to nitrogen atmosphere followed by further incubation at 50 °C for 30 min. After adding 400 μL of buffer (10 mM TrisHCl, pH 7.5; 150 mM NaCl; 2 mM EDTA), the reactions were incubated at 50 °C for 30 min, vortexed vigorously, and sonicated in a water bath for 15 min. After adding 100 μL of apoLp-III from a 5 mg/mL stock solution, vesicles were formed during an overnight incubation at 24 °C. After negative staining with 2% phosphotungstate, the apoLp-III-DMPC vesicles were visualized by transmission electron microscopy (Philips CM 120 Biotwin).

Low-Density Lipoprotein (LDL) Protection Assay. Lipid-binding properties of apoLp-III and the apoLp-III mutants were compared by the LDL-turbidity assay outlined in detail previously (*22*). Phospholipase C (PL-C)-induced aggregation of LDL leading to increase in turbidity of the suspension can be measured photometrically. In presence of apoLp-III, LDL aggregation is prevented. Fifty micrograms of human LDL suspended in 50 mM TrisHCl, pH 7.5, containing 150 mM NaCl and 2 mM $CaCl_2$ were incubated with 160 milliunits of phospholipase C (PL-C, Sigma P 7147) in the presence of 50 μg of apoLp-III or each of the apoLp-III-mutants. Reactions were performed at 37 °C in a total volume of 200 μL in a 96 well plate for $4^1/_4$ hours and absorbance was measured photometrically at 405 nm every 10−15 min using a 96-well plate-reader. Control reactions were performed in the absence of PL-C or apoLp-III.

Immune Activation. Immune inducing activity of apoLp-III and of the apoLp-III-mutants was measured 24 h after dorsolateral intrahemocoelic injection of 10 μL of a 5 mg/mL protein solution dissolved in sterile distilled water using 1 mL syringes and 20-gauges needles (Luer). The cuticle was punctured in the intersegmental region between two abdominal segments. Prior to injection solutions were centrifuged at 14 000 rpm for 5 min. Control injections were performed with sterile distilled water and bovine serum albumine (BSA, Sigma A 2934, 50 μg in 10 μL of sterile water). Twenty-four hours after injection, one of the first prolegs was pierced, the outflowing hemolymph was collected in capillaries and immediately transferred to Eppendorf tubes. A few crystals of phenylthiourea were added to prevent melanization. Hemolymph samples were centrifuged at 13 000 rpm for 5 min and the cell free supernatant stored at −20 °C prior to determination of antibacterial activity.

Determination of Antibacterial Activity. Antibacterial activity of hemolymph samples against lipopolysaccharide-defective and streptomycin- and ampicillin-resistant *E. coli* (K 12 strain D31) was determined by an inhibition zone assay described previously by Faye and Wyatt (*23*). Lytic activity against freeze-dried *Micrococcus luteus* (Sigma) was measured by a lytic zone assay (*24*) modified according to Wiesner (*25*). Test plates were incubated for 24 h after application of 3 μL of each of the cell-free hemolymph samples. The assays were standardized with gentamycin (Sigma) and hen egg-white lysozyme (Sigma), respectively. Significance of data was calculated with the Mann−Whitney rank sum test using SigmaStat 2.03 (Jandel scientific) as software. Level of significance (P-value) was set at less than 0.001.

RESULTS

Site-Directed Mutagenesis of ApoLp-III. A nucleotide exchange from A to G at position 196 in wild-type apoLp-III cDNA led to the mutant apoLp-III(N66D). ApoLp-III-(K68E) was generated by a nucleotide exchange from A to G at position 202. A double mutant apoLp-III(N66D/K68E) was created by the two nucleotide exchanges indicated above. The successful mutagenesis was confirmed by DNA-sequencing. Nucleotide sequences at all other positions were identical to the wild-type apoLp-III cDNA. On the basis of the structural data obtained from *M. sexta* apoLp-III by NMR spectroscopy and assuming a similar conformation for the *G. mellonella* protein, mutation of the *G. mellonella* apoLp-III at amino acid positions 66 and 68 might affect the putative hinge region between helices 2 and 3 (Figure 1).

The purified proteins appeared as single bands in a Coomassie Brilliant Blue stained polyacrylamide gel (Figure 2). MALDI-MS analyses of the mutant proteins showed single peaks indicating a molecular mass of 18 075.1 Da for

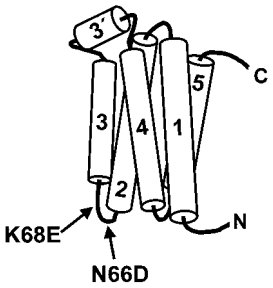

FIGURE 1: Schematic representation of the tertiary structure of apoLp-III in the globular conformation. The figure was adopted from the *M. sexta* apoLp-III. The three-dimensional structure of this protein may serve as a model valid for all lepidopteran apoLp-IIIs whose primary structure is very similar. The hydrophopic surface of five amphipathic α-helices are oriented toward the proteins' interior. The α-helices 3 and 4 are connected by an additional minihelix-3′ lying outside the bundle. According to the model, the amino acid substitutions in *G. mellonella* apoLp-III indicated by arrows affect the loop between helices 2 and 3.

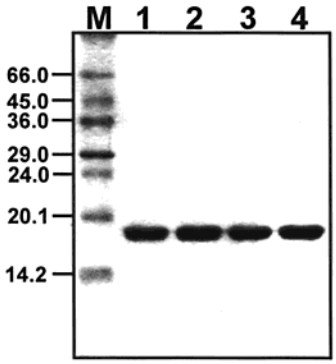

FIGURE 2: Purification of the proteins. The figure shows a 15% polyacrylamide gel stained with Coomassie Brillant blue G 250. M = marker proteins; lane 1 = wild-type apoLp-III; lane 2 = apoLp-III(N66D); lane 3 = apoLp-III(K68E); lane 4 = apoLp-III-(N66D/K68E). Each lane contained 4 μg of purified protein. Each preparation shows a high degree of purity, which was additionally confirmed by MALDI-TOF mass spectrometry (see Table 1).

wild-type apoLp-III, 18 078.0 Da for apoLp-III(N66D), 18 076.6 Da for apoLp-III(K68E), and 18 077.2 Da for apoLp-III(N66D/K68E). These data are in are in accordance with the calculated masses (Table 1). In summary, SDS−PAGE analysis and mass spectrometry data confirm the high degree of purity of all proteins used in this study.

Structural Characterization of Wild-Type ApoLp-III and the Mutant Proteins. Far UV-circular dichroism data indicated that in comparison to the α-helical content of wild-type apoLp-III (78%) the mutations introduced in the hinge region lead to a reduction of α-helical content of the single mutant apoLp-IIIs [61% for apoLp-III(N66D); 60% for apoLp-III(K68E)]. Structural data calculated for the double mutant (76% α-helical content) do only slightly differ from those of the wild-type apoLp-III (Table 1). Decrease in α-helical content of the single mutant apoLp-IIIs is accompanied by an increase in percentage amount of β-turns from 2% for the wild-type protein to 16% and 18% for apoLp-III(K68E) and apoLp-III(N66D), respectively. Stability of secondary structure determined by heat denaturation revealed similar denaturation midpoint temperatures of all proteins ranging between 54.7 °C for apoLp-III(N66D/K68E) and 57.12 °C for wild-type apoLp-III (Table 1).

The ability of the proteins to form complexes with DMPC was assessed to determine their size and shape after visualization by electron microscopy. Possible differences could indicate differences in lipid-binding ability as well as in protein folding in the lipid associated conformation. Electron micrographs of DMPC-apoLp-III complexes are shown in Figure 3. DMPC vesicles prepared with wild-type apoLp-III (Figure 3A) were similar in size and shape in comparison to complexes formed by the mutant proteins (Figure 3, panels B−D). ApoLp-III-DMPC-vesicles were about 18−20 nm in diameter indicating that the number of apoLp-III and the mutant proteins per vesicle are identical.

Comparison of Binding Affinity of apoLp-III and Mutant ApoLp-IIIs to Destabilized LDL. Although no changes in size and shape of DMPC complexes prepared with the mutant apoLp-IIIs could be detected, significant differences were observed regarding the binding affinity of the proteins to destabilized LDL particles. Figure 4A shows the results of LDL protection assays. Increase in turbidity of the LDL suspension in the presence of 50 μg of wild-type apoLp-III and 160 milliunits of PL-C was almost completely abolished during a time span of 255 min. When reactions were performed with apoLp-III(N66D/K68E) or apoLp-III(K68E) a strong and fast increase in absorbance was detected, which indicates a substantial reduction of lipid-binding ability of these two mutants. ApoLp-III(N66D) prevented LDL aggregation only to a somewhat less efficiently extent than the wild-type protein. Figure 4B shows LDL protection assays performed in the presence of wild-type apoLp-III or apoLp-III(N66D/K68E). In these experiments, PL-C was inhibited by adding 250 mM KBr 60 min after PL-C-induced LDL aggregation had been started. Inhibition of PL-C in the presence of apoLp-III(N66D/K68E) was followed by a faster increase in turbidity than it was observed for wild-type protein. After 255 min, turbidity of PL-C inhibited LDL aggregation in the absence of any apoLp-III and in the presence of the double mutant increases to the same extent. Thus, reduction of the lipid-binding ability of apoLp-III-(N66D/K68E) might be caused by a poor stability of lipid binding rather than by a decreased initiation of lipid binding.

Immune Inducing Activity of ApoLp-III in Comparison to the Mutant Proteins. As shown in Figure 5, injection of wild-type apoLp-III results in a substantial increase of antimicrobial activities of the hemolymph. It was of interest, therefore, to test whether the introduced mutations also affected—beside lipid binding—the immune stimulating function.

Injection of apoLp-III(N66D/K68E) had a significant effect on the extent of immune induction. Both hemolymph lysozyme activity (panel A) and anti-*E. coli* activity (panel B) reach only 56 and 63%, respectively, of those detected after injection of wild-type apoLp-III. The apoLp-III(K68E) also exhibited a significantly reduced immune-activating effect, at least as detected by the anti-*E. coli* activity. In comparison to wild-type apoLp-III, immune stimulation of apoLp-III(N66D) was unaffected.

DISCUSSION

The results of the present study provide the first evidence that the immune activating function of apoLp-III depends on its ability to bind to lipid surfaces. Amino acid substitutions that led to decreased lipid association of the protein

Table 1: Biophysical Properties of Wild-Type ApoLp-III and the Mutant Proteins

	mass data		structural data				stability
	calculated mass (Da)	MALDI-MS analysis (Da)	% α-helix	% β-sheet	% β-turn	% remainder	heat denaturation midpoint (°C)
wild-type apoLp-III	18 075.0	18 075.1	78	−	2	20	57.12
apoLp-III(N66D)	18 076.0	18 078.0	61	−	18	21	56.03
apoLp-III(K68E)	18 075.9	18 076.6	60	−	16	24	55.11
apoLp-III(N66D/K68E)	18 076.9	18 077.2	76	−	1	23	54.70

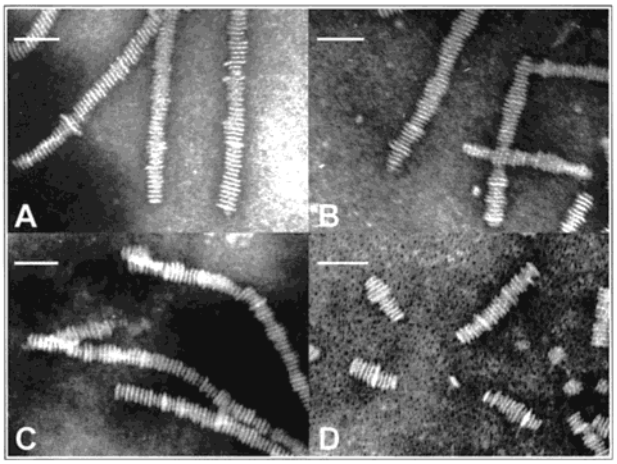

FIGURE 3: Electron micrographs of apoLp-III-DMPC-complexes. The complexes were prepared as outlined in the experimental procedures in the presence of wild-type apoLp-III (A), apoLp-III-(N66D) (B), apoLp-III(K68E) (C), and apoLp-III(N66D/K68E) (D), respectively. Negative staining was performed with 2% phosphotungstate. The apoLp-III-DMPC-complexes are equal in size and shape. Each bar represents 50 nm.

affected likewise the immune-stimulating properties of apoLp-III. Although in the double mutant protein apoLp-III(N66D/K68E) no significant alterations of physical parameters such as secondary structure or stability against heat denaturation were detected, both lipid association and immune inducing activities were severely affected. The hypothesis that lipid binding is required for apoLp-III to exert its immune stimulating function is further supported by the experiments using the apoLp-III with single amino acid substitutions. For example, the reduction of lipid association observed with apoLp-III(K68E) was less pronounced than in the double mutant protein, and this difference was paralleled by the different ability of this protein to induce antibacterial activities in the hemolymph.

The establishment of a bacterial expression system for *G. mellonella* apoLp-III provided a powerful tool to study the function of the protein by site-directed mutagenesis, which in a series of reports has also been performed with apoLp-III of *L. migratoria* and *M. sexta*. As most studies focused on the mode of action of lipid binding (*5*, *6*), they revealed valuable information about the regions within the protein, which enable its unfolding upon formation of LDLp (*26*−*28*). Studies on apoLp-III *of M. sexta*, whose primary structure is very similar to that of *G. mellonella*, demonstrated that the protein can be "frozen" in its globular conformation after introducing a disulfide bridge coupling the loop regions between helices 1 and 2 and helices 3 and 4. As apoLp-III opening of the oxidized protein was prevented, the loops between helices 2 and 3 and helices 4 and 5 were believed to act as hinges (*15*). The present study

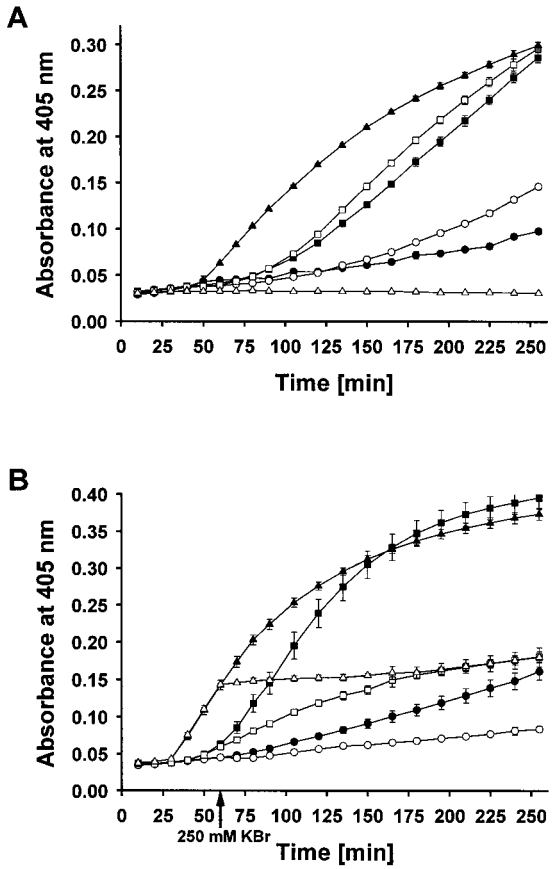

FIGURE 4: Protection of LDL against PL-C induced aggregation by apoLp-III and the apoLp-III-mutants. (A) Human LDL (50 μg of protein) was incubated with 160 milliunits of PL-C at 37 °C in the presence of wild-type apoLp-III (●) and the mutant forms, apoLp-III [N66D (○)], apoLp-III [K68E (■)], or apoLp-III [N66D/K68E (□)]. Control reactions were performed either in the absence of apolipoprotein (▲) or PL-C (△). Turbidity was measured photometrically at 405 nm. (B) LDL protection against PL-C-induced aggregation in the presence of wild-type apoLp-III (●, ○) apoLp-III(N66D/K68E; ■, □) or in the absence of apolipoprotein (▲, △). Sample incubations were the same as in panel A; however, curves with white symbols represent incubations, where PL-C-induced LDL aggregation was inhibited by adding KBr (250 mM final concentration) after 60 min. Data are given as mean ± standard deviation of three determinations.

is the first investigation demonstrating functional consequences of site-directed mutagenesis directly within one of these putative hinge regions. Especially, the mutant apoLp-IIIs (K68E) and (N66D/K68E) displayed interesting differences in their lipid-binding ability.

A comparison of the amino acid residues within the putative hinge between helices 2 and 3 of the *G. mellonella* apoLp-III (ANGKA, positions 65−69) with homologous lepidopteran proteins indicates a high degree of similarity. Variable residues can only be found at the second position, where a serine residue is found in *Agrius convolvuli* apoLp-

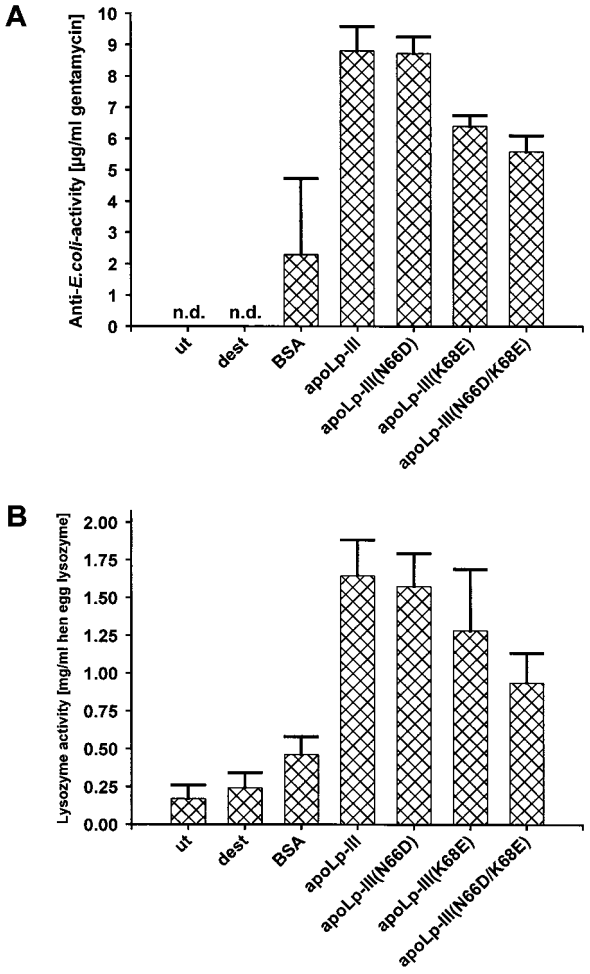

FIGURE 5: Immune activation by wild-type apoLp-III in comparison to the mutant apoLp-IIIs. Twenty-four hours after injection of apoLp-III, apoLp-III(N66D), apoLp-III(K68E), or apoLp-III(N66D/K68E) (50 μg/larva), antibacterial activity against *E. coli* (A) was measured by using an inhibition zone assay standardized with gentamycin. (B) Lysozyme activity was detected by a lytic zone assay with hen egg lysozyme as standard. The tested sample volume was 3 μL each. Data are given as mean $\pm$ standard deviation; $n = 10$; n.d., not detectable.

III (GenBank accession no. AF001632.1), and at the third position, where an alanine exists in *Spodoptera litura* apoLp-III (GenBank accession no. AF094582.1). The lysine residue, however, whose substitution turned out to have major effects on lipid association and immune activation is conserved within all known lepidopteran apoLp-IIIs. Therefore, it appeared reasonable to introduce the mutations N66D and/or K68E into the *G. mellonella* apoLp-III and to compare their consequences on lipid binding and immune activation. They fulfilled the prerequisites of minimal sterical changes concerning the size of the substituted amino acids and maximal changes in charges. As lipid binding of apoLp-III to lipolyzed LDL is believed to function by hydrophobic interactions rather than by electrostatic forces (*6*), loss of lipid-binding ability of apoLp-III(N66D/K68E) and apoLp-III(K68E) (Figure 4A) is likely to be caused by a reduced stability of lipid association. This suggestion is confirmed by the experiments shown in Figure 4B. After inhibition of PL-C a faster increase in turbidity by LDL aggregation is observed when the reaction is performed with the double mutant protein as compared to the wild-type apoLp-III. This indicates that mutagenesis of the putative hinge region performed in this study does not affect the initiation of lipid association, but might reduce the stability of the protein−lipid complex. Consequently, these data confirm the idea of the loop between α-helices 2 and 3 to function as hinge. They indicate that the lysine residue at position 68 might be essential for accurate hinge function.

The structural data obtained from UV CD spectroscopy and the electron micrographs of DMPC vesicles demonstrate that the effects of the mutations on lipid binding cannot be due to major changes in secondary structure neither in the lipid free nor in the lipid associated conformation. The proteins' ability to form discoidal complexes of equal size and shape with DMPC (Figure 3), indicate that—once associated with lipids—the structure of the mutant apoLp-IIIs in the lipid associated conformation does not differ from the wild-type protein. Although α-helical contents of the single mutant proteins in the lipid free conformation are slightly reduced, it appears unlikely that the effects on lipid binding are caused by significant changes in the molecular architecture of the mutant apoLp-IIIs. Especially, the double mutant protein is very similar to wild-type apoLp-III regarding secondary structure. Moreover, the stability of all mutant apoLp-IIIs against heat denaturation was not affected by mutagenesis.

The observation that a reduced lipid association of apoLp-III decreases its immune stimulating activity may help to understand the long-standing problem regarding its function in larval hemolymph. Namely, it may serve as an important molecule for the mediation of immune responses. The constitutive existence of apoLp-III in larval hemolymph and the negligible increase of soluble apoLp-III following intrahemocoelic injection (*12*, *13*) excluded the possibility of cytokine- or hormon-like properties of apoLp-III. Rather, it suggests that immune activation by apoLp-III might be caused by a conformational change of the protein, which normally does not occur in larval hemolymph (*8*). The data of the present study document that intrahemocoelic injection of wild-type apoLp-III may increase the lipid-bound fraction of the protein, which might be the signal for immune activation. The evidences for LDLp formation after bacterial immune provocation (Dettloff et al., submitted for publication) and uptake of lipid-bound apoLp-III by hemocytes (*29*) support this idea. Thus, the mechanism of apoLp-III-induced immune activation might be triggered by hemocyte uptake of LDLp provoking them to release further immune mediating factors.

Assuming this mechanism, the results shown in Figure 4B indicate, that decrease in immune inducing activity of apoLp-III(N66D/K68E) might be caused by an inefficient ability to form stable LDLp during "immune-related" LDLp biogenesis.

Finally, the evidence for a relationship between lipid-binding and immune-inducing properties of apoLp-III establishes the role of this protein as a mediator of insect immune responses. This observation provides a rational explanation for the constitutive existence of apoLp-III in larval hemolymph in a globular conformation. With respect to these findings it is worth mentioning that the structural homologue of apoLp-III in vertebrates, apo E, is also suspected to play a role in mammalian immunology (*30*). Thus, a close relationship between lipid physiology and

immunology might be postulated to be valid for a wide variety of animal species ranging from insects to mammals.

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