Structural Studies of Lipid Binding Proteins

Deborah R. Breiter, Matthew M. Benning, Michael R. Kanost,* Michael A. Wells,* John H. Law,* Ivan Rayment, and Hazel M. Holden

*Departments of Chemistry and Biochemistry and the Institute for Enzyme Research, University of Wisconsin-Madison, Madison, Wisconsin; *Department of Biochemistry, University of Arizona, Tucson, Arizona*

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ABSTRACT

The three-dimensional structures of two different insect lipid transport proteins have been solved by x-ray crystallographic analyses: apolipophorin-III (apoLp-III) isolated from *Locusta migratoria* and insecticyanin isolated from *Manduca sexta* L..

In the case of insecticyanin, its molecular structure has been solved and refined to a nominal resolution of 2.0 Å. Interestingly, the overall three-dimensional architecture of the molecule shows remarkable similarity to the structural motifs exhibited by bovine β-lactoglobulin and human serum retinol-binding protein. The three-dimensional fold of insecticyanin devoid of its biliverdin chromophore has also been determined to 2.2 Å resolution.

With respect to apoLp-III, its structure has recently been solved to a nominal resolution of 2.5 Å. As predicted from amino acid sequence studies, the secondary structure of this protein is almost entirely α-helical with five long amphiphilic helices. The preliminary structural investigation of apoLp-III described here is of special importance since it represents the first successful high resolution x-ray analysis of an apolipoprotein.

Taken together, the x-ray crystallographic investigations described in this chapter have provided important structural information on two very different classes of insect lipid binding proteins. Furthermore, while the focus is on insect models, the knowledge gained from these studies will undoubtedly provide valuable insight into mammalian lipid transport pathways as well.

INTRODUCTION

Lipids serve both structural and metabolic roles in all living systems. Consequently, the effective transport of such water insoluble molecules is of central biochemical importance. Both vertebrate and invertebrate species have evolved a variety of methods to overcome the problem of shuttling hydrophobic molecules within an aqueous environment. For example, in both mammals and insects, there exist soluble lipoprotein particles specifically designed for the transport of lipids in the blood or hemolymph. These large lipoprotein complexes are believed to be composed of a lipid core surrounded

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by a "coat" of protein. Another type of lipid transport in both mammalian and insect systems are the small proteins, such as retinol binding protein, fatty acid binding protein and insecticyanin, that bind only one lipid molecule thereby rendering it soluble in an aqueous medium.

In lipoprotein complexes, the lipids and proteins are held together primarily by hydrophobic effects and their geometric relationship may not be rigidly defined. In contrast, proteins such as insecticyanin bind one lipid molecule in a precise and specific manner. We are presently investigating, by x-ray crystallographic techniques, the molecular structures of two insect lipid binding proteins; apolipophorin-III (apoLp-III) from *Locusta migratoria* and insecticyanin from *Manduca sexta* L.. These proteins were chosen for study because they represent specific examples of the above-mentioned classes. ApoLp-III is part of a lipoprotein complex whereas insecticyanin belongs to the category of small lipid-binding proteins. As will be described, these two proteins differ markedly in their three-dimensional architecture and thus provide excellent models for understanding the different structural elements involved in protein:lipid interactions. Insect models were chosen for these structural studies because their lipid transport systems are somewhat less complicated than their mammalian counterparts. However, it is important to note that insects use lipids for many of the same processes as do vertebrates and consequently what is learned from these x-ray investigations will also yield valuable insight into the mammalian lipid transport pathways.

I. INSECTICYANIN

Insecticyanin is a blue biliprotein isolated from the tobacco hornworm, *Manduca sexta* L.. Although its biological role is not entirely clear, insecticyanin is believed to play a key role in the camouflage coloration of the insect [1]. The protein was first isolated and purified by Cherbas [2] and the amino acid sequence subsequently determined by Riley *et al.*, [3]. Each subunit of insecticyanin contains 189 amino acid residues with two disulfide bridges. In terms of quaternary structure, insecticyanin is a tetramer with 222 symmetry as seen in the crystalline lattice [4]. The chromophore responsible for giving insecticyanin its intense blue coloration is the γ-isomer of biliverdin IX as first proposed by Cherbas [2] and demonstrated by crystallographic analyses [4].

The molecular structure of insecticyanin was initially solved to a resolution of 2.6 Å using the techniques of multiple isomorphous replacement and molecular averaging [4]. Least squares refinement of the model to a nominal resolution of 2.0 Å resolution has now been completed with a final crystallographic R-factor of 15.7% using x-ray data from 5.0 Å to 2.0 Å resolution. Details of the refinement will be published elsewhere. An α-carbon trace of one subunit of insecticyanin, along with the biliverdin, is shown in stereo on the following page.

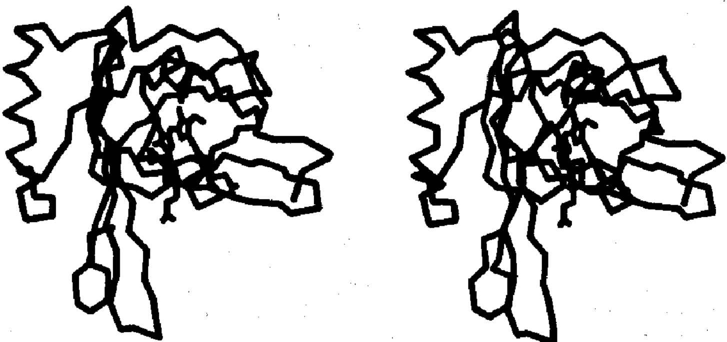

FIG. 1. *α-carbon trace of insecticyanin.*

The individual subunit shown above has overall dimensions of 44 Å x 37 Å x 40 Å and consists primarily of an eight-stranded anti-parallel β-barrel flanked on one side by a 4.5-turn α-helix. The biliverdin lies towards the open end of the β-barrel with its two propionate side chains pointing towards the solvent and it adopts a rather folded conformation, much like a heme.

As the initial polypeptide model for insecticyanin was being built, it became apparent that the overall three-dimensional fold of the molecule was remarkably similar to the structures of bovine β-lactoglobulin [5], and human serum retinol-binding protein [6]. Dr. Terry Meyer at the University of Arizona has carried out amino acid sequence alignments of these proteins based on three-dimensional considerations and has found there to be less than a dozen common residues (personal communication). Yet, when considering only the positions of the alpha carbons, insecticyanin and human serum retinol binding protein superimposed with an RMS value of approximately 1.6 Å for 93 structurally equivalent atoms. For the superposition of bovine β-lactoglobulin and insecticyanin, the RMS value for a total of 86 structurally equivalent α-carbon positions is approximately 2.0 Å.

Within the last several years it has become increasingly apparent that this molecular fold seen in insecticyanin, bovine β-lactoglobulin, and human serum retinol binding protein may be more common than was originally thought. A variant of this structural motif has now been crystallographically demonstrated in rat intestinal fatty acid binding protein [7], bovine P2 myelin protein [8] and photoreceptor protein [9]. These proteins contain a ten-stranded rather than eight stranded anti-parallel β-barrel. Amino acid sequence studies have further suggested that a variety of proteins with quite different physiological functions may have three-dimensional structures similar to that observed in insecticyanin, examples of which include human α_1-microglobulin [10], olfactory binding protein [11,12], rat α_{2u}-globulin, [13], human α_1-acid glycoprotein [14], human placental protein 14 [15], rat androgen-dependent secretory protein [16], purpurin [17] and human apolipoprotein D [18]. Apolipoprotein D is of particular interest since it has been assumed that this apolipoprotein binds and transports cholesterol [18,19]. Recent model building and binding studies of Peitsch and Boguski

[20], however, suggest that apolipoprotein D binds bilirubin and other heme-related metabolites. Clearly, the structural motif seen in insecticyanin is ideally suited for the binding and transport of small, hydrophobic molecules and undoubtedly will be found in still other proteins whose biological role is to transport such ligands.

In an effort to understand on a molecular level the specificity of the insecticyanin binding pocket as well as the conformational flexibility of the protein fold we have solved the structure of the protein devoid of biliverdin as described in the next section.

II. APOINSECTICYANIN

The biliverdin chromophore is tightly bound to insecticyanin and can only be removed under denaturing conditions such as treatment with formamide [2] or guanidine hydrochloride [3]. Following the procedure of Cherbas [2], apoinsecticyanin was prepared by treatment with 50% formamide, followed by column chromatography to separate the protein and ligand. The formamide was removed by extensive dialysis against 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 150 mM NaCl and 5 mM sodium azide, pH 7.5. Details of the protein preparation, crystallization, x-ray structure determination and least squares refinement will be published elsewhere. In summary, the apoinsecticyanin structure was solved by the technique of molecular replacement and the model is presently being refined to a nominal resolution of 2.2 Å. The current R-factor is 22.0% with good overall molecular geometry. An α-carbon trace of one subunit of apoinsecticyanin superimposed on the native structure is shown in stereo below.

FIG. 2. *Superposition of insecticyanin and apoinsecticyanin.*

The apo-structure is shown above in bold lines; the native insecticyanin model along with the biliverdin ligand is shown in thin lines. As can be seen the overall molecular fold has changed very little except for one loop region near the opening of the binding pocket. While a detailed comparison of these two models must await completion of the refinement of apoinsecticyanin, it can be concluded that their overall three-dimensional architecture is very similar.

As a continuing effort to understand the conformational flexibility of this protein fold, experiments have been initiated to substitute the biliverdin with other small hydrophobic ligands. Small amber-colored crystals of insecticyanin in which the biliverdin has been replaced by heme have now been grown. Experiments designed to bind other molecules in the insecticyanin pocket are in progress.

III. APOLIPOPHORIN III

Insecticyanin is one of the major hemolymph proteins found in the tobacco hornworm as well in the adult sphinx moth. There is another major component in the hemolymph of the adult moth referred to as lipophorin. Lipophorin is a lipoprotein particle and depending on the metabolic state of the insect, is typically comprised of 60% protein and 40% lipid [21]. The protein portion of the lipophorin particle consists of two apolipoproteins referred to as apolipophorin I and apolipophorin II. Both apolipophorin I and apolipophorin II are integral constituents of lipophorin and have molecular weights of approximately 250,000 and 80,000 respectively [22,23]. In some insects there exists another apolipoprotein referred to apolipophorin III (apoLp-III). This protein typically has a molecular weight of approximately 20,000 and exists as a soluble monomer in the hemolymph. During flight, however, when lipophorin is loaded with diacylglycerol, apoLp-III associates with the lipoprotein particle and it has been suggested that this smaller apoprotein serves to stabilize the lipid-enriched complex [24,25,26].

ApoLp-III has been isolated from several insect species but the most extensively studied proteins are those isolated from *Manduca sexta* and *Locusta migratoria*. It has been suggested that the *M. sexta* protein is a prolate ellipsoid with an axial ratio of approximately 3 based on its hydrodynamic properties and its behavior during gel permeation chromatography [25]. In addition, the circular dichroic spectrum of apoLp-III indicates a helical content of approximately 50%. The amino acid sequence of the sphinx moth apoLp-III, deduced from the cDNA sequence, shows the presence of repeating tetradecapeptide units, each having the potential to form amphiphilic helices [27]. A sequence homology study of apoLp-III with mammalian apolipoproteins suggests considerable functional but little amino acid sequence identity [27].

Locust apoLp-III was first studied by Van der Horst *et al.*, [28] and subsequently purified to homogeneity by Chino and Yazawa [29]. Unlike the *M. sexta* protein, however, this apoLp-III is glycosylated with a total sugar content of approximately 12%. A cDNA coding for the locust protein has recently been cloned and the amino acid sequence determined based on the cDNA sequence [30]. The protein contains 161 amino acid residues and sequence comparisons with the *M. sexta* molecule have revealed a homology of approximately 30%.

Although there is little amino acid sequence identity between apoLp-IIIs isolated from different insects, the surface properties of these small apolipoproteins may be more critical than the actual sequence identity in fulfilling their physiological function. Support for this view is found in the studies of Van der Horst *et al.*, [28]. Their experiments demonstrate that the uptake of lipids by lipophorin in an *in vitro* fat body preparation from *L. migratoria* is supported equally well using apoLp-III from either *L.*

migratoria or *M. sexta*. Clearly, speculations about similar surface properties can only be confirmed when the three-dimensional structures of the various proteins are known.

Due to the small size of apoLp-III and the fact that during certain metabolic states it exists as a soluble hemolymph protein, it seemed an ideal apolipoprotein for crystallization trials. Small micro-crystals of apoLp-III isolated from the adult sphinx moth were grown but all attempts to prepare larger crystals for an x-ray analysis were unsuccessful. However, the apolipoprotein isolated from the African locust, *Locusta migratoria*, crystallized readily and in a form suitable for a high resolution structural analysis [31].

The molecular structure of locust apoLp-III described here was determined by multiple isomorphous replacement using six heavy atom derivatives. Details of the structure determination will be described elsewhere (manuscript in preparation). An electron density map calculated to a nominal resolution of 3.0 Å was used to build the initial model and the structure is presently being refined to a resolution of 2.5 Å. The first six amino acid residues as well as residues 157 to 161 are not clear in the electron density map. Otherwise, the amino acid sequence has been fitted unambiguously into the electron density. A ribbon drawing of this apolipoprotein is shown below.

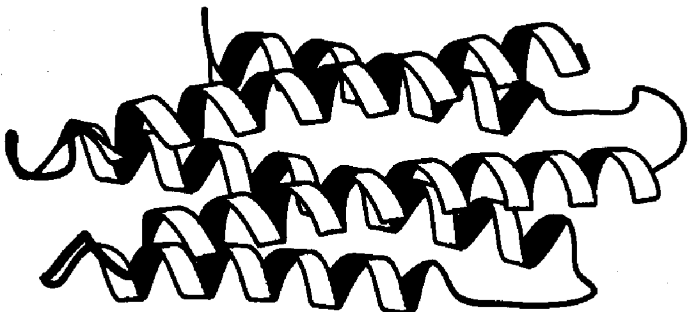

FIG. 3. *Ribbon drawing of apoLp-III.*

As can be seen from the figure, the protein contains five long amphiphilic helices. Three of these helices order up to 30 amino acid residues while the other two helices contain approximately 20 amino acid residues. The molecule is indeed elongated as first suggested by Kawooya *et al.*, [25] with an approximate length of 53 Å and a width of 22 Å.

Locust apoLp-III can exist as either a soluble monomer in the hemolymph of the insect or bound to lipophorin. Numerous biophysical and biochemical studies indicate that apoLp-III undergoes a profound conformational change upon binding to the lipoprotein complex [25]. Since the crystals used in this investigation were grown in the absence of any lipid [31], the next step in the structural analysis of locust apoLp-III is to grow crystals in the presence of lipids and/or detergents. These experiments are presently underway.

There have been many investigations within recent years directed toward understanding the structural elements of apolipoproteins that allow them to interact with lipid surfaces. It has been suggested that amphiphilic helices play a key role in such protein:lipid interactions [32]. To date, however, there has been a lack of three-dimensional structural information concerning apolipoproteins and these putative helices. The molecular structure of apoLp-III described here is the first apolipoprotein structure to be solved by x-ray crystallographic techniques and represents the first direct three-dimensional evidence for the presence of amphiphilic helices in this class of lipid transport proteins. Also, while the primary sequence homology between insect apolipoproteins and mammalian apolipoproteins is low, as in the case of insecticyanin and human serum retinol binding protein, it is likely that their overall three-dimensional structures are quite similar.

In conclusion, the molecular structures of two insect proteins involved in lipid transport have been solved by x-ray crystallographic techniques. Insecticyanin represents one class of lipid transport proteins that binds a hydrophobic ligand in a well defined 1:1 ratio. The overall molecular structure of this molecule can be described simply as an eight-stranded anti-parallel β-barrel flanked on one side by a 4.5 turn α-helix. ApoLp-III belongs to another class, the apolipoproteins, which interact with a lipoprotein particle in a still not well understood manner. Unlike insecticyanin which contains predominantly β-sheet, apoLp-III is almost entirely α-helical. These two different structural motifs have clearly evolved to overcome the problem of shuttling hydrophobic molecules within the aqueous milieu of the organism.

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