A new class of hexahelical insect proteins revealed as putative carriers of small hydrophobic ligands

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Background: THP12 is an abundant and extraordinarily hydrophilic hemolymph protein from the mealworm *Tenebrio molitor* and belongs to a group of small insect proteins with four highly conserved cysteine residues. Despite their sequence homology to odorant-binding proteins and pheromone-binding proteins, the function of these proteins is unclear.

Results: The first three-dimensional structure of THP12 has been determined by multidimensional NMR spectroscopy. The protein has a nonbundle helical structure consisting of six α helices. The arrangement of the α helices has a 'baseball glove' shape. In addition to the hydrophobic core, electrostatic interactions make contributions to the overall stability of the protein. NMR binding studies demonstrated the binding of small hydrophobic ligands to the single hydrophobic groove in THP12. Comparing the structure of THP12 with the predicted secondary structure of homologs reveals a common fold for this new class of insect proteins. A search with the program DALI revealed extensive similarity between the three-dimensional structure of THP12 and the N-terminal domain (residues 1–95) of recoverin, a member of the family of calcium-binding EF-hand proteins.

Conclusions: Although the biological function of this new class of proteins is as yet undetermined, a general role as α-helical carrier proteins for small hydrophobic ligands, such as fatty acids or pheromones, is proposed on the basis of NMR-shift perturbation spectroscopy.

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Introduction

Insects and terrestrial arthropods comprise over 80% of terrestrial life forms. Because of their rapid reproductive capability, insects are major competitors with humans for natural food resources. In this regard, many billions of dollars are lost annually from the actions of insect pests. Insects can also provide valuable information for the study of those biochemical processes that they share with mammals [1]. The incredible variation among insect species complicates the identification and functional characterization of insect proteins, however. An important step towards simplifying this problem is to recognize homologies both in the primary sequence and at the structural level between various proteins, followed by protein classification. To date, however, the search for structural homologies has been complicated by the fact that of nearly 8000 solved protein structures only ten are of insect proteins. Therefore, solving the protein structure of selected class members offers an efficient way to reduce the expenditure on X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy of individual proteins and provides a way to gain more insight into the function of unknown proteins.

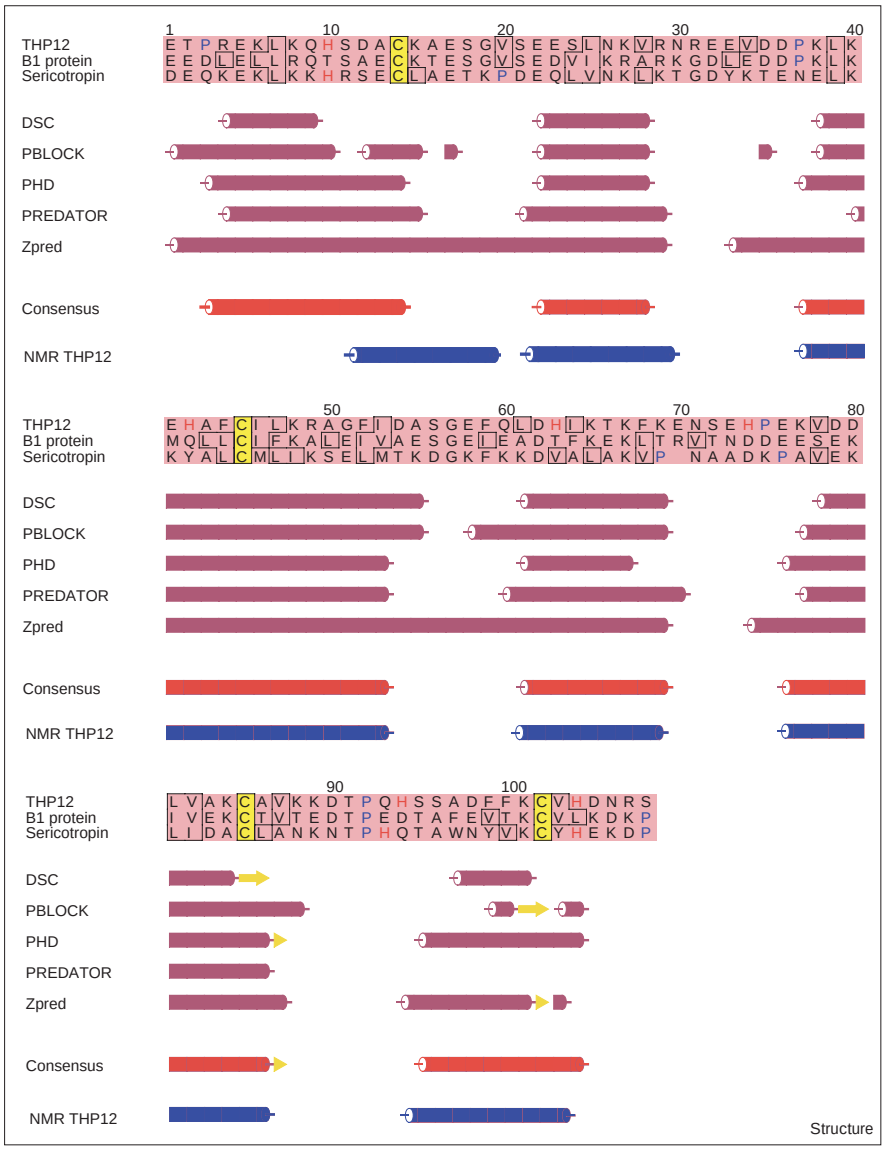
Previously, we succeeded in expressing an abundant 12.4 kDa hemolymph protein (THP12) from mealworm beetle *Tenebrio molitor* as a representative of a new class of insect proteins, and reported the secondary structure and backbone dynamics [2]. In the present paper, we describe for the first time the tertiary structure of THP12. We also report the results of NMR binding studies employed to find common structural features in this class of proteins, with the ultimate aim of achieving a better understanding of their biological function.

Results and discussion

Sequence homology

THP12 and three other proteins (two B proteins and sericotropin) that are distributed in different insect tissues show significant sequence similarities, including four conserved cysteine residues (Figure 1). B proteins represent one of the major protein groups secreted from the tubular accessory glands of the adult male mealworm beetle [3], whereas sericotropin is a secretory protein that was identified in wax moth larvae (*Galleria mellonella*) and is released from the brain [3]. A role as a carrier for small hydrophobic ligands has been suggested for B proteins and sericotropin on the basis of their weak sequence homology to a group of pheromone-binding proteins (PBPs) and odorant-binding proteins (OBPs) [3–5]. OBPs and PBPs belong to the lipocalin-type superfamily. The

Figure 1



Amino acid sequence alignment of THP12, B1 protein and sericotropin. The numbering is shown for THP12 only. Conserved cysteine residues are highlighted in yellow, proline and histidine residues are shown in blue and red, respectively, and aliphatic amino acids are outlined in black. Secondary structures were analyzed using the consensus secondary structure prediction software JPred [8]. JPred runs the predictions DSC, PBLOCK, PHD, PREDATOR and Zpred for building the consensus (red cylinders). The positions of the α helices in the NMR solution structure of THP12 are indicated as blue cylinders.

basic structural motif of lipocalin-type proteins comprises ten antiparallel β strands, which form a β barrel, and two short α helices [6,7].

The three-dimensional structure of THP12

For the structure determination of THP12, ^{1}H,^{15}N chemical-shift assignments [2] were confirmed and complimented by essentially complete ^{13}C-resonance assignments. The tertiary structure of the protein was primarily determined with large numbers of nuclear Overhauser effect (NOE) restraints derived from three-dimensional ^{13}C-edited NOE spectra (3D ^{1}H–^{13}C NOESY-HSQC) from a ^{13}C/^{15}N double-labeled sample as well as dihedral angle and J-coupling restraints. It should be noted that due to missing long-range NOEs in ^{15}N-NOESY-HSQC experiments, double-labeling was essential for building the folding model of THP12. Figure 2 shows the location and mostly even distribution of long-range NOEs between the helices. The structure can be summarized as a nonbundle α-helical structure consisting of six α helices with loops between the helices (Figure 3) and a disordered N terminus (residues 1–10). The hydrophobic core of the protein is formed by helices D, E and F, which lie approximately parallel to each other, and helix C oriented perpendicular to the others. The aromatic residues Phe52, Phe68, Phe99 and Phe100 are clustered in the hydrophobic core. This arrangement of helices is strongly supported by long-range NOEs between the residues Val28–His104, Phe44–Gln93, Phe44–Ser96, Ile64–Val82, Phe68–Val78, Leu81–Val103, Ala86–Ile64,

and slowly exchanging amide protons of residues Ala43–Asp54, Ile64 and Val82–Val87 of helices C, D and E. The close proximity of helices A and B and of helices E and F is due to the formation of the disulfide bonds Cys14–Cys45 and Cys85–Cys102, respectively. Additional stability is contributed by electrostatic interactions between Arg31 and Asp105, Glu76 and Lys69 and Glu79 and Lys65. The fact that THP12 is relatively heat-stable (transition midpoint $T_M = 67^oC$) yet its structural stability is highly pH-dependent are consequences of these interactions. Circular dichroism studies revealed a complete loss of helicity on changing the pH from 5.0 to 3.0 (Figure 4). Nevertheless, the majority of positively and negatively charged residues are evenly distributed on the protein surface (Figure 5a), rendering it very soluble in aqueous solution. Overall, the structural core of the protein formed by the six α helices is well defined with an average backbone root mean square deviation (rmsd) of 0.62 Å between 23 (converged out of 50) calculated structures and the mean structure. A summary of the structural statistics for the set of final structures is listed in Table 1.

Structure homology

The secondary structures of B proteins and sericotropin, predicted (Figure 1) using the program JPred [8], were found to be remarkably similar to the α-helical structure found for THP12 [2]. Moreover, the alignment of structurally important residues such as prolines and glycines in loop regions, together with the prediction of six α-helical domains, strongly suggests a similar fold for this new class of proteins. However, the lack of primary structure similarity and differences in secondary or tertiary structure highlight an apparent discrepancy in the suggested relationship to PBPs and OBPs. Although THP12, B proteins, and sericotropin comprise a mainly α-helical structure, lipocalins form β-barrel structures. Furthermore, Du and Prestwich [9] have described a somewhat higher helical content (> 45%) in insect PBPs, larger than that for vertebrate PBPs, suggesting that two different protein folds might serve the same function. Thus a role of these proteins as general carrier proteins for small hydrophobic ligands should not be excluded. When only limited information regarding sequence homology to functionally known proteins is available, it is instructive to search for structural similarities to known protein folds. Such a search with the program DALI [10] unveiled extensive similarity between the three-dimensional structure of THP12 and the N-terminal domain (residues 1–95) of recoverin (Figure 6). Inspection of the biological function of recoverin provides an interesting comparison. Recoverin belongs to a group of Ca^{2+}-binding proteins that consist of multiple pairs of the helix-loop-helix motif (EF-hand). It was found that in the calcium-free state, the N-terminally attached myristoyl group is sequestered in a hydrophobic cavity of recoverin. Interestingly, the binding pocket is formed by the five N-terminal helices [11], which show the highest structural similarity to THP12. However, no significant sequence homology can be observed between recoverin and THP12. In this regard, THP12 lacks the typical sequence motif of calcium-coordinating residues in the EF-hands, thus Ca^{2+} binding can be excluded. On the other hand, similar requirements for binding to small ligands such as fatty acids can be expected for both proteins on the basis of structural homology and comparable distributions of hydrophobic and hydrophilic residues. In THP12, a number of hydrophobic and particularly aromatic residues, such as Phe52, Ile53, Phe59 and Phe60, can be found in equivalent steric positions to residues Ile52, Tyr53, Phe56 and Phe57 of recoverin, although the sidechains originate from different residues (again highlighting the lack of similarity at the sequence level).

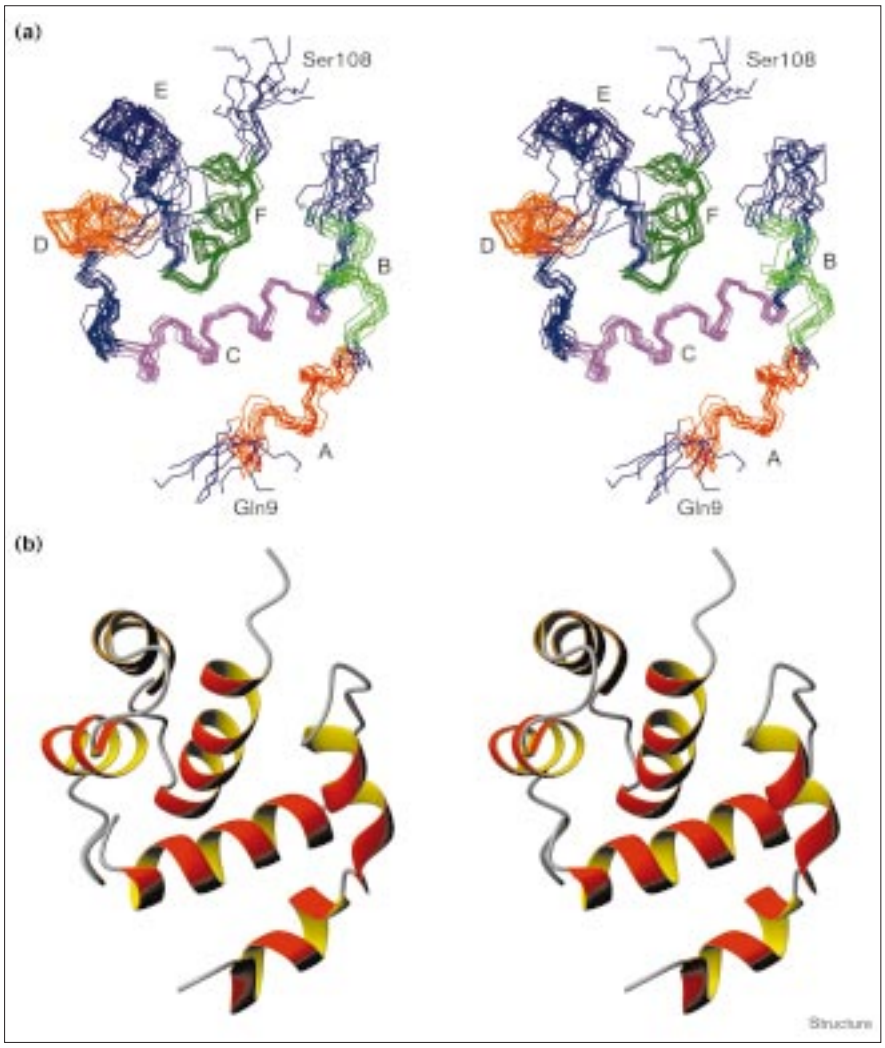
Figure 2

The three-dimensional structure of THP12. The structure is shown as a backbone trace (N, Cα, C′) and the location of long-range NOEs (|i–j| > 5 residues) are shown as thick black lines. (The figure was generated with the program Insight II.)

Functional studies

To gain more insight into the binding capabilities of THP12, size-exclusion chromatography (SEC) and subsequent liquid chromatography/mass spectroscopy (LC-MS), NMR spectroscopy and H/D exchange methods were employed. NMR-shift perturbation experiments [12], namely titration of the ^{15}N-labeled THP12 with solutions of the fatty acids nonanoic acid and octanoic acid and

Figure 3

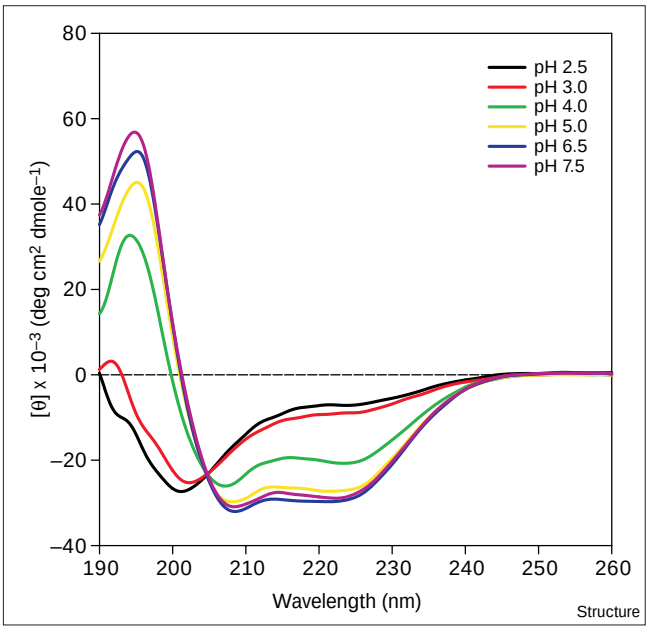


Stereoviews of the overall fold of THP12. (a) Best-fit backbone superposition of the NMR structures of THP12. The backbone atoms of residues 9–108 are overlayed. For clarity, eight structures were chosen out of a total of 23. Helices A, B, C, D, E and F are colored red, green, purple, orange, dark blue and dark green, respectively. (b) Schematic ribbon drawing of THP12. Secondary structure elements are indicated. (The figures were generated using the program MOLMOL [33].)

the *T. molitor* pheromone 4-methylnonanol, also showed significant binding (Figure 7). Regions of large chemical-shift perturbations in two-dimensional ^{15}N-HSQC spectra were determined for residues Thr66, Phe68, Lys69, Glu70, His74 and Ala97 after the addition of ligands. These residues flank the single hydrophobic groove in THP12 (Figure 5b). As discussed by many authors, conformational flexibility is important for ligand binding and in particular for the binding of ligands in the central cavity of a protein [13–15]. In this regard, chemical-shift changes at residues Thr66–His74 suggest a conformational shift of the loop connecting helices D and E away from the protein bulk as a result of ligand interaction. Moreover, participation of the loop in ligand binding supports the hypothesis of the presence of a ligand entry portal [16–18] involving the loop between helices D and E, similar to that found in fatty acid binding proteins. The dimensions of the open hydrophobic groove appear large enough to admit access of different kinds of small apolar ligands without steric conflicts. Thus, no additional conformational changes need to occur. Referring again to recoverin, one notes that the location of the putative hydrophobic binding pocket in THP12 corresponds to that found in recoverin. In both cases, the cavity or groove is formed by aromatic and other hydrophobic residues contributed by the four or five flanking helices.

SEC fractionation and molecule identification by LC-MS were used to screen endogenous ligands in the water-soluble fraction of hemolymph from *T. molitor* larvae. These studies revealed some evidence for ergosterol (MW 396.6) as an additional ligand, producing identical HPLC retention times on co-injection of commercial ergosterol. As changes in protection against H/D exchange are expected to be sensitive to the bound or non-bound states of tight protein–ligand complexes, we

Figure 4



The pH dependence of circular dichroism spectra in the pH range 2.5–7.5 for THP12.

measured the kinetics of H/D exchange for THP12 in the presence and absence of the ligands ergosterol and 4-methylnonanol by mass spectrometry. The free protein shows 22 protected protons, but within the experimental error of the method no additional protection was observed for the ergosterol and 4-methylnonanol complexes. However, this observation indicating weak binding does not conflict with the proposed function of this protein. It is generally thought that carrier proteins share several biological functions. Besides protection of biologically relevant molecules against degradation, these include nonspecific transportation and the delivery of different types of ligands to destinations such as receptors. Thus, the determined low ligand affinity in the millimolar range and lower specificity compared with receptors should fulfill the requirements for general carrier protein complexes from which biologically relevant molecules could rapidly dissociate.

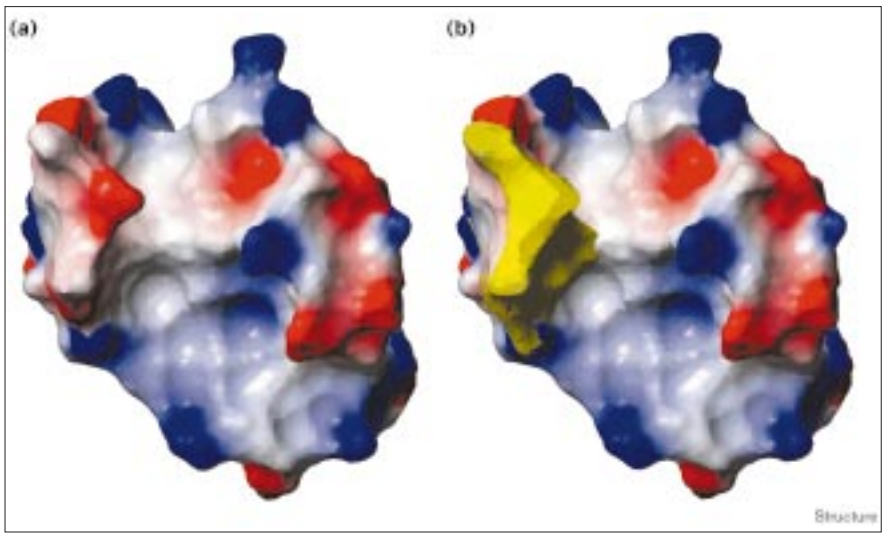
Biological implications

Insects and terrestrial arthropods comprise over 80% of terrestrial life forms. Because of their rapid reproductive capability, insects are major competitors with humans for natural food resources. In this regard, many billions of dollars are lost annually from the actions of insect pests. Insects can also provide valuable information for the study of those biochemical processes that they share with mammals.

We present here the first three-dimensional structure of THP12, a 108 amino acid protein from the hemolymph of the yellow meal worm *Tenebrio molitor*. The structure of THP12 is described as a nonbundle helical structure consisting of six α helices. Our results lend strong support to the idea that THP12 is a new member of a class of hexahelical proteins, which have been described as sharing a general carrier function. Moreover, the high solubility of this class of proteins in aqueous solution is ideal for the binding and nonspecific transportation of small hydrophobic ligands through hydrophilic media. Sequence and structure homology between these proteins together with the observed binding of the pheromone 4-methylnonanol to THP12 support the proposed function [3] of B proteins (also members of this

Figure 5

Molecular surface of THP12. (a) Distribution of the electrostatic potential on the surface of THP12 (residues 9–108). The view is in the same orientation as in Figure 2. The positive potential is shown in blue, the negative potential is in red. (b) Residues with chemical-shift perturbations after titration with the pheromone 4-methylnonanol are colored in yellow. (The figure was generated with the program MOLMOL [33].)



family) in transporting pheromones to membrane receptors. The proposed function of these proteins is also supported by the extensive similarity between the three-dimensional structure of THP12 and the N-terminal domain (residues 1–95) of recoverin. Recoverin belongs to a group of Ca^{2+}-binding proteins and binds an N-terminally attached myristoyl group in a hydrophobic cavity. The binding pocket is formed by five N-terminal helices, which show high structural similarity to THP12. In contrast to H/D exchange studies monitored by mass spectrometry or NMR spectroscopy, NMR-based shift perturbation spectroscopy is the method of choice for the discovery of potential ligands of general carrier proteins, particularly in the case of low ligand affinities (micromolar to millimolar) or slow binding kinetics.

Table 1

Structure statistics for the 23 best structures of THP12 after simulated annealing and energy minimization.

Restraints for structure calculations		
Total restraints	1517	
Total NOE restraints	1370	
intraresidue	645	
sequential	276	
medium-range	204	
long-range	245	
Dihedral angle constraints	84	
J-coupling restraints	63	
Statistics for structure calculations		
Rmsd from idealized geometry	<SA>	<SA_{min}>
bonds (Å)	0.0021 ± 0.0001	0.0020
bond angles (º)	0.440 ± 0.011	0.430
improper torsions (º)	0.270 ± 0.005	0.269
NOEs*	0.0062 ± 0.001	0.0068
dihedral angle restraints*	0.16 ± 0.097	0.25
J-coupling constraints	0.55 ± 0.040	0.56
Final energies (kcal mol^{-1})		
E_{total}	137.5 ± 8.3	129.9
E_{bonds}	7.6 ± 0.8	6.7
E_{angles}	91.9 ± 4.2	87.7
$E_{improper}$	9.7 ± 0.3	9.6
E_{vdW}	24.8 ± 3.0	22.2
E_{NOE}	2.7 ± 0.9	3.1
$E_{dihedral}$	0.2 ± 0.1	0.3
$E_{J\ coupling}$	0.7 ± 0.2	0.1
Coordinate precision (Å)	<SA> vs <SA_{av}>	<SA_{min}> vs <SA_{av}>
Rmsd of backbone atoms†		
for residues 11–105	0.82 ± 0.12	0.46
for core regions‡	0.60 ± 0.07	0.39
Rmsd of all heavy atoms		
for residues 11–105	1.31 ± 0.13	0.74
for core regions‡	1.01 ± 0.07	0.64
Ramachandran plot statistics§		
most favored regions (%)		80.5
additional allowed regions (%)		17.2
generously allowed regions (%)		2.3
disallowed regions (%)		0

*None of the final structures included in the ensemble had distance restraint violations greater than 0.3 Å or dihedral angle restraint violations in excess of 1°. †N, Cα, C′. ‡Residues 13–28, 36–39 and 77–103. §As determined by PROCHECK [34].

Materials and methods

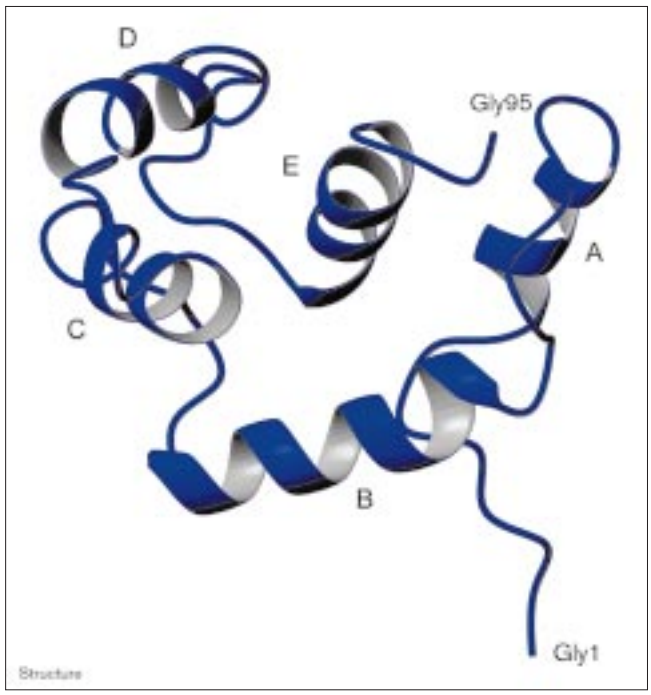
Sample preparation

The expression of recombinant THP12 has been described previously [2]. For labeling with ^{15}N and ^{13}C isotopes, THP12 was overexpressed at 37°C in minimal medium containing 50 mM Na_2HPO_4, 20 mM KH_2PO_4, 8.5 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.8% ^{13}C glucose and 0.05% $(^{15}NH_4)_2SO_4$. Protein production was induced using 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) when the cell density reached an OD_{600} of 0.8. After induction for 3 h, cells were collected by centrifugation at 3000 × g for 20 min and were resuspended in buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.1 mM PMSF) for sonication at 0°C. The sonicate was centrifuged at 12,000 × g for 25 min, and the supernatant fraction was then loaded onto a Sephadex G-75 column (3.0 × 100 cm). The A_{230} value of the elution profile was measured, and the location of THP12 was determined by Tris-Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Pooled fractions containing THP12 were lyophilized and resuspended in 0.1% trifluoroacetic acid (TFA) (buffer A) for further purification by reversed-phase HPLC on a C_{18} preparative column (2.2 × 25 cm). THP12 was eluted using a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 8 ml/min, and came off the column at 32% acetonitrile. The mass of the protein was confirmed by electrospray ionization mass spectrometry (ESI).

NMR spectroscopy and structure calculation

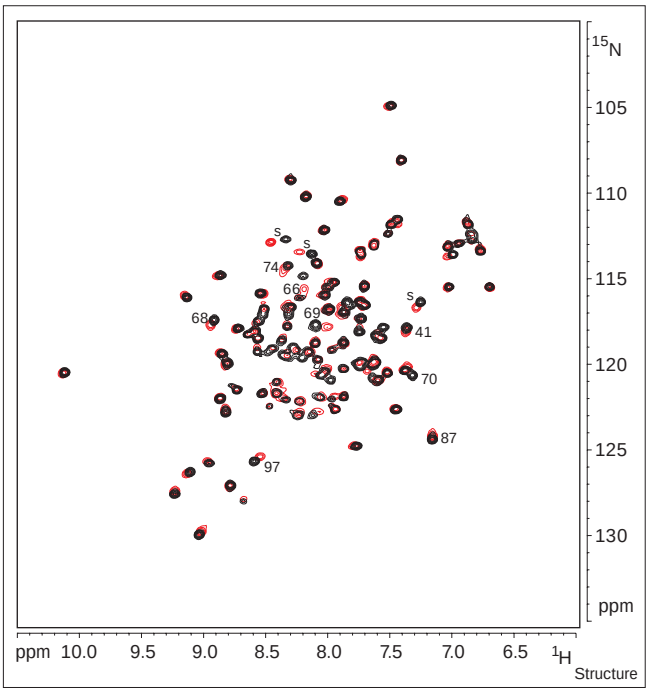
NMR measurements were made on a 1 mM protein sample in 95% H_2O/5% D_2O at pH 6.9. NMR experiments were recorded on Varian UNITY Plus 600 MHz and INOVA 500 MHz spectrometers equipped with pulsed-field gradient units and probes with an actively shielded z gradient. ^{15}N-based experiments have been described [2]. Backbone sequential resonances were completed using HNCACB [19] and CBCA(CO)NH [20] experiments. The sidechain resonances were assigned using 3D ^{15}N-TOCSY-HSQC [21], 3D HCCH-TOCSY [22] and 3D C(CO)NH-TOCSY [23]. Stereospecific assignments were obtained from a combination of HNHA [24], HNHB [25], and short

Figure 6



Schematic ribbon drawing of the N-terminal domain (residues 1–95) of recoverin [11]. (The figure was generated with the program MOLMOL [33].)

Figure 7



^{1}H–^{15}N heteronuclear single-quantum correlated NMR spectra (measured at 600 MHz) of THP12 (25°C, pH 6.9). Spectra are shown in the absence (black) and presence (red) of the pheromone 4-methylnonanol.

time ^{15}N-TOCSY-HSQC [21] experiments. NMR data were analyzed using the programs NMRPipe [26] and PIPP [27].

Distance restraints for the structure calculation were collected from 2D and 3D ^{15}N- or ^{13}C-edited NOESY experiments and 4D ^{13}C and ^{13}C-edited NOESY experiments [28–30] and converted into distances based on their intensities using residue-based calibrations as described previously [31]. Structures were calculated using standard spectropolarimeter distance geometry (DG) and simulated-annealing protocols implemented in the program X-PLOR 3.81 [32].

Circular dichroism studies

Circular dichroism spectra were recorded on a Jasco J-720 spectropolarimeter (Jasco, Eaton, MD). A water bath was used to control the temperature of the cuvette. The pH dependence of spectra was measured by varying the pH in the range 2.5–7.5. The protein was dissolved in water to a final concentration of 1.0×10^{-5} M as determined by amino acid analysis.

Functional tests

In total, 200 µl hemolymph were collected from 100 adult mealworms, rinsed in distilled water containing 10 mM protease inhibitor Pefabloc (Merck) and centrifuged (6000 g, 2 min). The aqueous fraction (100 µl) was injected onto the SEC column Superdex peptide HR 10/30 (Pharmacia Biotech). Fractions between 10 and 15 kDa, which contained THP12, were taken for LC-MS studies. Reversed-phase HPLC separations were carried out on a Vydac C_{18} column (150 × 1 mm ID, 5 µm). Gradient elution was performed at an eluent flow rate of 30 µl/min using a linear gradient of 7–80% B over a period of 40 min (mobile phases A and B consisted of water containing 0.05% TFA and acetonitrile-water [8:2, v/v] containing 0.05% TFA, respectively). The total flow was divided at the outlet of the column by means of a zero-dead-volume tee resulting in the introduction of 4 µl in a UV detector (785A, Applied Biosystems) with micro cell type ZU (LC packings) operating at 220 nm. The second arm of the tee was connected via a fused-silica capillary directly to the electrospray interface (flow rate 26 µl/min). Mass spectrometry was performed on a triple quadrupole instrument (TSQ 700, Finnigan MAT, Bremen, Germany) equipped with an electrospray ion source (API-ESI) operating in the positive mode with a capillary temperature of 200°C and a high voltage of 4.5 kV. Nitrogen was introduced as sheath gas at a pressure of 3.4 bar and auxiliary gas (960 ml/min). A sheath liquid was applied of isopropanol-propionic acid (25:75, v/v) at a flow rate of 4 µl/min.

NMR binding studies were performed as chemical-shift perturbation experiments by titration of 1 mM uniformly ^{15}N-labeled protein samples with ligand solutions at pH 6.9. The titrations were carried out in steps by adding 10 µl of 10 mM ligand solutions. For the analysis of intermolecular interactions, the ^{15}N-HSQC spectrum in the presence of the ligand was compared with that of the free protein.

H/D exchange studies

ESI mass spectrometry was performed on a triple quadrupole instrument (TSQ 700, Finnigan MAT, Bremen, Germany) equipped with an electrospray ion source (API-ESI) operating in the positive mode with a capillary temperature of 200°C and a high voltage of 5 kV. The sample concentration was 200 pmol/µl in 10 mM ammonium acetate buffer (pH 6.0). H/D exchange was initiated by diluting the sample 100-fold into D_2O. The sample was then immediately introduced into the ion source at a constant flow rate of 4 µl/min. Nitrogen was used as sheath gas at a pressure of 4.8 bar. The mass difference between deuterated protein and non-deuterated protein gives the average of incorporated deuterium atoms. For binding studies, two ratios 1:1 and 1:10 (c/c) of protein to ligand were applied.

Accession numbers

The coordinates have been deposited at the Protein Data Bank under the accession numbers 1C3Y and 13CZ for the ensemble of structures and the minimized average structure, respectively.

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