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Binding properties of a locust's chemosensory protein☆

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Abstract

The chemosensory protein CSP-*sg*4 of the desert locust *Schistocerca gregaria* binds reversibly *N*-phenyl-1-naphthylamine in fluorescent-binding assays, with a dissociation constant of 4 μM. Upon binding to the protein, the emission peaks of the fluorescent probe undergo a marked blue shift, accompanied by an order of magnitude increase of the maximum intensity. The assay has also allowed the measurement of the affinity of CSP to other aromatic and aliphatic compounds. The binding capacity of this protein is unaffected by thermal treatments up to 100 °C for 20 min. The ligand-binding characteristics of chemosensory proteins may help in clarifying the role of this recently discovered class of soluble proteins in chemoreception. © 2002 Elsevier Science (USA). All rights reserved.

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Chemoreception in insects is mediated by G protein-coupled receptors, recently discovered in *Drosophila melanogaster* [1,2]. However, soluble proteins, capable of reversibly binding pheromones and odorants, seem also to be involved in the perception of semiochemicals. These proteins, that are highly concentrated in the lymph of chemosensilla, belong to two distinct classes of polypeptides, named OBPs (odorant-binding proteins) and CSPs (chemosensory proteins) [3–7]. OBPs, further divided into PBPs (pheromone-binding proteins) and GOBPs (general odorant-binding proteins), contain about 140 residues and share six conserved cysteines, connected by three interlocking disulphide bridges [8,9]. A large number of OBPs have been described in insects of different orders, each species expressing several members of both subclasses. The structure of *Bombyx mori* PBP has been resolved and the position of its ligand, bombykol, has been identified inside the binding cavity. It consists mainly of α-helical domains that confer high stability to the molecule [10].

Ligand-binding studies have been performed since the discovery of PBPs, using radioactively labelled pheromones [3]. Although the binding activity of these proteins towards their specific pheromones has been clearly demonstrated, the methods used in the past did not allow a clear estimate of the relative dissociation constants. Recently, however, a more reliable method has been described that allows measurement of binding constants at the equilibrium [11]. This assay utilises the fluorescent ligand 1-aminoanthracene, previously employed with success with vertebrates' OBPs [12].

CSPs are also widely expressed among insects and better conserved than OBPs across evolution [13–20]. They are shorter than OBPs (about 110 residues) and contain only four cysteine residues, linked by two non-interlocked disulfide bridges [7]. Preliminary NMR data indicate that, as observed with OBPs, also CSPs are constituted mainly by α-helical domains, but their three-dimensional structure has not yet been resolved [21,22]. The role of CSPs in chemoreception is mainly based on their high concentration in the lymph of chemosensilla, but the lack of binding data has so far prevented a clear definition of their physiological function. In locusts

☆ *Abbreviations:* CSP, chemosensory protein; GOBP, general odorant-binding protein; NPN, *N*-phenylnaphthylamine; OBP, odorant-binding protein; PBP, pheromone-binding protein; THP, tetrahydropyranyl.

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several sequences of CSPs, belonging to different subclasses, have been reported [7,20]. They are expressed in sensory hairs of antennae, legs, and palpi, as well as in cells underlying the cuticle. These data have suggested a function of such proteins both in the perception and the delivery of chemical signals [7].

Here we report for the first time ligand-binding data for a recombinant CSP of the desert locust *Schistocerca gregaria*, obtained through a simple and reliable fluorescent assay, a contribution towards the elucidation of the function of CSPs in insect's chemical communication.

Materials and methods

Protein synthesis and purification. The protein used in this study, chemosensory protein CSP-*sg*4 of *S. gregaria* [7], was obtained by bacterial expression, as previously described [21]. This method produced the polypeptide in high yield (20 mg/l of culture), bearing an initial methionine residue as the sole modification with respect to the native protein. The recombinant CSP, that was exclusively present in soluble form, was purified by a combination of gel filtration and anion-exchange chromatography, as previously described, and the correct pairing of the two disulfide bridges was verified by mass spectrometry [21].

Synthesis of ligands. *N*-Benzylnaphthylamine was prepared by reacting 10 mmol of 1-naphthylamine with 10 mmol of benzaldehyde in 50 ml of methanol and in the presence of 5 mmol of sodium cyanoborohydride. Tetrahydropyranyl (THP) ethers of 1-hexanol and phenol were prepared, as previously described, by reaction of the above compounds with 2-dihydropyrane [23]. 1,2-Dipyrazylethane was prepared by treatment of pyrazine with butyllithium, followed by reaction with 1,2-dibromoethane. All other ligands are commercially available.

Fluorescence measurements. Emission fluorescence spectra were recorded on a Jasco FP-750 instrument at 25° C in a right angle configuration with a 1 cm light path quartz cuvette and 5 nm slits for both excitation and emission. The protein was dissolved in 50 mM Tris buffer, pH 7.4, while ligands were added as 1 mM methanol solutions.

Intrinsic fluorescence. The tryptophan intrinsic fluorescence was measured on a 5 μM solution of the protein, using an excitation wavelength of 295 nm and recording the emission spectrum between 300 and 360 nm. Quenching of intrinsic fluorescence was measured in the same condition and in the presence of *N*-phenyl-1-naphthylamine (1-NPN) at concentrations of 1 and 20 μM.

Binding assays. To measure the affinity of the fluorescent ligand 1-NPN to CSP, a 5 μM solution of protein in 50 mM Tris, pH 7.4, was titrated with aliquots of 1 mM methanol solutions of the ligand to final concentrations of 1–20 μM. The affinity of other ligands was measured in competitive binding assays, using 1-NPN as the fluorescent reporter at 5 μM concentration and concentrations of 2–300 μM for each competitor.

Temperature stability measurements. One ml aliquots of 5 μM CSP in Tris buffer were kept for 5, 10, and 20 min at temperatures of 75, 85, 95, and 100 °C. After cooling to room temperature, 5 μl of 1 mM methanol solution of 1-NPN was added and fluorescence spectra were recorded between 380 and 560 nm, using an excitation wavelength of 337 nm.

Data analysis. For determining binding constants, the intensity values corresponding to the maximum of fluorescence emission were plotted against free ligand concentrations. Bound ligand was evaluated from the values of fluorescence intensity assuming that the protein was 100% active, with a stoichiometry of 1:1 protein:ligand at saturation. The curves were linearised using Scatchard plots. Dissociation constants of the competitors were calculated from the corresponding IC_{50} values, using the equation: $K_D = [IC_{50}]/(1 + [1\text{-NPN}]/K_{1\text{-NPN}})$, [1-NPN] being the free concentration of 1-NPN and $K_{1\text{-NPN}}$ being the dissociation constant of the complex CSP/1-NPN.

Results and discussion

The chemosensory protein of *S. gregaria*, used in this study (CSP-*sg*4), has a single tryptophan at position 81 of the mature protein. When excited at 295 nm, this residue produces a fluorescence peak with its maximum at 330 nm (Fig. 1, curve A), indicating a rather hydrophobic environment and suggesting that the tryptophan is located inside the core of the protein.

To find a suitable fluorescent reporter for CSP, we tried several compounds commercially available. Among these, 1-NPN had been reported to undergo a strong blue shift with emission at 395 nm and an increase in the intensity of fluorescence when bound to lipocalins, such as OBPs and MUPs (major urinary proteins) [24]. In fact, when excited at 337 nm, 1-NPN produces a very weak fluorescent peak at 480 nm (Fig. 2, curve A). In the presence of CSP, the emission wavelength is shifted to 407 nm, accompanied by a 10-fold increase in the intensity (Fig. 2, curve B). The intensity of this peak was used to measure the amount of 1-NPN bound to the protein. Such binding is saturable with a single population of binding sites, without apparent cooperativity effect, and a dissociation constant of 4 μM (Fig. 3A and B). Its isomer 2-NPN also binds in a similar way, but with much lower affinity (data not shown).

To verify that 1-NPN was actually bound inside the protein, rather than interacting on its surface, the quenching of intrinsic fluorescence of the protein was

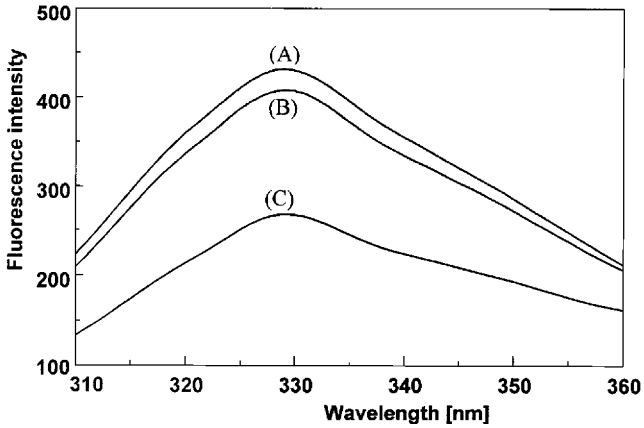

Fig. 1. Intrinsic fluorescence of CSP. Trp81, when excited at the wavelength of 295 nm gave an emission peak at 330 nm, indicating a rather hydrophobic environment for this amino acid (curve A). In the presence of 1-NPN at concentrations of 1 μM (curve B) and 20 μM (curve C) the fluorescence emission of tryptophan is quenched, indicating that the probe binds inside the binding pocket. Protein concentration was 5 μM in Tris buffer, pH 7.4.

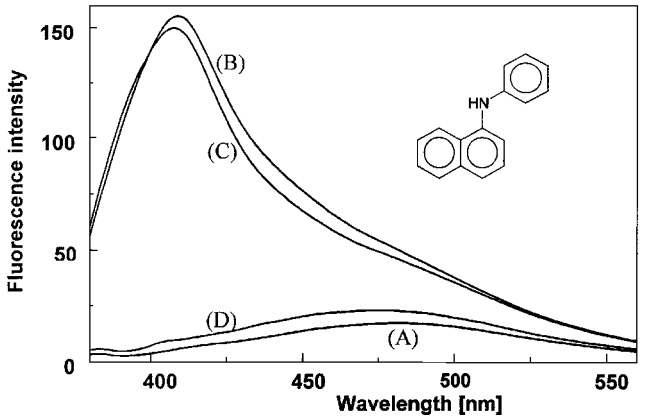

Fig. 2. Fluorescence emission spectrum of 1-NPN 5 μM in Tris buffer excited at the wavelength of 337 nm. (A): 1-NPN alone; (B) 1-NPN + CSP, both at the concentration of 5 μM; (C) as in (B), but with a sample of CSP kept at 100 °C for 20 min; (D) as in (B), but in the presence of 4 M guanidium chloride.

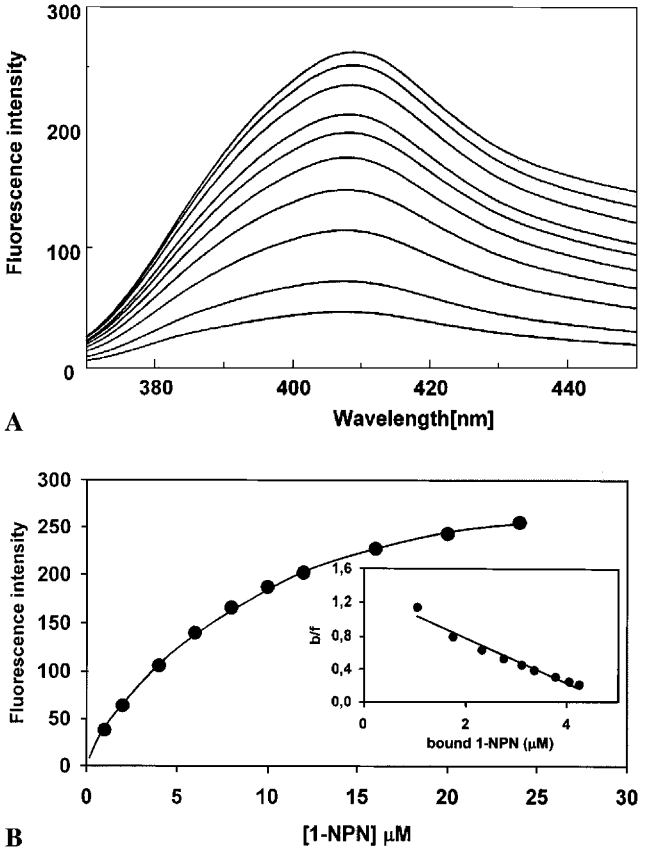

Fig. 3. Binding of 1-NPN to CSP. Protein was 5 μM in Tris buffer, pH 7.4. Aliquots of a 1 mM methanol solution of 1-NPN were added to the protein to final concentrations of 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, and 28 μM and emission spectra were recorded (A). The binding curve and the relative Scathard plot (B) indicate a binding constant of 4 μM.

measured in the presence of 1-NPN at concentrations of 1 μM (Fig. 1, curve B) and 20 μM (Fig. 1, curve C). In fact, a quenching effect is observed with a decrease of the tryptophan fluorescence intensity down to 60% of the starting value.

To further support the assumption that the fluorescence peak at 407 nm is relative to a specific interaction of 1-NPN within a binding cavity, we verified that such a peak is completely absent when 1-NPN is incubated with a sample of the protein denatured in 4 M guanidinium chloride (Fig. 2, curve D).

We investigated the thermal stability of CSP by measuring the intensity of the emission peak at 407 nm. Spectra were therefore recorded with the protein incubated at temperatures up to 100 °C for up to 20 min, as described in the Materials and methods section. All the samples, after cooling to room temperature, fully recovered their binding activity. Fig. 2, curve B reports the emission spectrum of CSP treated at 100 °C for 20 min. Such an exceptional stability of the functional protein is in agreement with the data of CD spectroscopy on the stability of its tertiary structure [21]. In fact, only temperatures as high as 90 °C caused partial unfolding observed in the CD spectra, and this was reversible when the protein was cooled down to 25°. Our present data further confirm such an unusual stability with reference to the binding function of the protein.

To investigate the specificity of the binding site of CSP, we have used a number of potential ligands either in direct fluorescent assays or in competitive binding experiments.

The first series included several fluorescent amines and phenols. 1-Aminoanthracene, that was successfully used in measuring the binding to vertebrates' OBPs [12] and more recently to insects' PBPs [22], failed to bind CSP. 1- and 2-naphthols, employed with MUPs [25], lipocalins structurally similar to OBPs, also failed to bind CSP. No binding was measured with either of the two naphthylamines. This behaviour, apparently surprising, particularly for the latter compounds, as compared to the good affinity of their phenyl derivatives 1-NPN and 2-NPN, was interpreted as a consequence of the higher basicity of naphthylamines as compared to their phenyl derivatives. Such a property makes 1-naphthylamine more hydrophilic than 1-NPN, enough to prevent this molecule from entering the binding cavity of CSP. The same argument could also explain the lack of binding observed with the relatively hydrophilic naphthols. To further support this hypothesis, we also tested *N*-benzyl-1-naphthylamine, failing to measure any binding. This molecule contains a methylene group as the sole structural difference with 1-NPN, but its basicity is much stronger than 1-NPN and comparable to that of 1-naphthylamine.

Since 1-NPN was the best ligand for CSP among those tested so far, we used this fluorescent probe in competitive binding experiments together with several potential ligands, belonging to different chemical and

Table 1
Affinities of different ligands to CSP-*sg*4, evaluated in competitive binding assays

Competitor	[IC_{50}] (μM)	K_D (μM)
3,7-Dimethyloctanol	100	44
1-Nonanol	130	58
Linalool	300	133
Citronellal	100	44
Citralva	300	133
Diphenylamine	280	124
2,2′-Dipyridyl	300	133
1,2-Dipyrazylethane	230	102
γ-Nonalactone	220	98
Cinnamaldehyde ethylene glycol acetal	75	33
2-Methoxycinnamaldehyde	55	24
2-Amylcinnamaldehyde	20	9

Protein (5 μM) was incubated with 5 μM 1-NPN. The intensity of the fluorescence emission peak at 407 nm was recorded in the presence of increasing concentrations of the competitor. Dissociation constants were calculated from the IC_{50} values, according to the formula: $K_D = [IC_{50}]/(1 + [1\text{-NPN}]/K_{1\text{-NPN}})$. The following compounds displaced less than 50% of 1-NPN from the complex at the highest concentration used of 300 μM: *cis*-3-hexen-1-ol, 3-phenyl-1-propanol, 2-phenylethanol, menthol, decanal, 2,2-diquinoline, phenylacetonitrile, anisole, guaiacol, *m*-cresol, *p*-methylanisole, 2,6-dimethylaniline, 2-methylpyrazine, phenol THP, 1-hexanol THP.

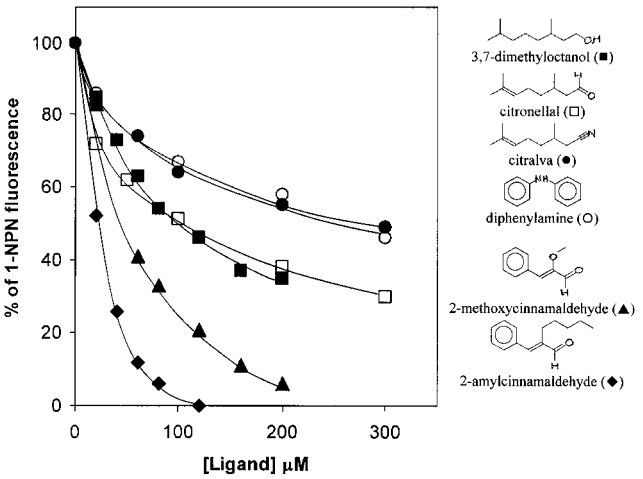

Fig. 4. Competitive binding of selected ligands to CSP. Protein (5 μM) was incubated with 1-NPN (5 μM) and aliquots of 10 mM solution of ligands were added to the final concentrations indicated. For each set of data, fluorescence values were plotted as percent of that obtained in the absence of competitor.

odour classes. The rapid and reliable fluorescent assay allowed a large screening of chemical compounds.

At the beginning we chose compounds reported to be pheromones for the studied species (*S. gregaria*) or for other species. These included phenylacetonitrile, anisole, and guaiacol, reported to produce aggregation in the desert locust [26]. Also carboxylic acids and linear alcohols of 12, 14, and 18 carbon atoms, as well as ethyl esters of the above acids, were tested. All these compounds failed to displace 1-NPN from its complex with CSP, even at ratios of 1:100. Another class of compounds, that could have been worth testing, is represented by long chain hydrocarbons, that have been recognised as sex pheromones in some species and have been suggested to mediate communication between individuals in social insects [27]. However, we could not use these potential ligands in our binding assays, due to their extremely low solubility in water.

Table 1 lists those compounds that could decrease by 50% the amount of bound 1-NPN at concentrations lower than 300 μM. Binding constants were calculated for these ligands and reported in the same table. The displacement curves, recorded for six representative ligands, are reported in Fig. 4, together with their relative structure. More compounds, that have been tested, but showed no binding affinity to CSP, are listed in the legend of Table 1.

None of the chemicals tested showed good affinity to CSP, the fluorescent probe 1-NPN still being the strongest ligand. Diphenylamine, which bears structural similarity to 1-NPN, exhibited some affinity, together with other compounds containing two aromaic rings, such as 2,2′-dipyridyl and 1,2-dipyrazylethane. Also flexible hydrophobic molecules, such as alcohols, aldehydes, and nitriles of 9–10 carbon atoms, could displace 1-NPN from the complex with CSP. These compounds, as well as derivatives of cinnamaldehyde, could mimic the bulk and shape of the fluorescent probe. The best ligand of Table 1 is 2-amylcinnamaldehyde, whose shape, if the structure is written in the appropriate conformation (Fig. 4), could reproduce that of 1-NPN. This information could be the starting basis for designing better ligands for CSP and get insights into the structures of natural ligands.

In conclusion, we have set up a simple and reliable binding assay at the equilibrium for insects' chemosensory proteins and defined some requirements for a good fitting of ligands to the proteins' binding sites. The three-dimensional structure of CSP, when available, will further clarify the interactions of the proteins of this class with small ligands.

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