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Analysis of the Silkworm Moth Pheromone Binding Protein – Pheromone Complex by Electrospray-Ionization Mass Spectrometry

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In the four decades since Butenandt et al. identified bombykol ((10*E*,12*Z*)-hexadeca-10,12-dien-1-ol) as the female sex attractant of the silkworm moth, *Bombyx mori*,[1] considerable progress has been made in our understanding of pheromone chemistry and biology. Throughout this time, the *B. mori* system has continued to serve as a useful model for unraveling the intricacies of chemical communication. Indeed, a complete picture is slowly emerging, from bombykol biosynthesis and regulation in females to olfactory detection and catabolism in male antennae.[2] The study of mechanisms involved in pheromone detection is particularly active, and has produced a detailed model of events at the molecular level. Lipophilic pheromone molecules enter the antennal sensilla (sensory hairs) through cuticular pores, where they come into contact with the aqueous lymph medium that surrounds the receptor (nerve) cells. Here they are solubilized by a pheromone binding protein (PBP; a small (< 20 kDa), water-soluble lipid binding protein),[3] and transported through the lymph to the nerve cell membrane. The pheromone receptor itself resides in this membrane, and there is strong evidence that it is G-protein-coupled.[4] Activation of the receptor, therefore, stimulates a secondary messenger cascade and ion-channel opening, thereby generating a receptor potential.

Progress towards total structural characterization of the *B. mori* PBP (BmPBP) has been rapid, from production of cDNA clones and deduction of the amino acid sequence,[5] to determination of the disulfide bridging pattern,[6] culminating in the elucidation of the X-ray crystal structure of BmPBP complexed with bombykol.[7] In an attempt to probe the interaction between pheromone and binding protein further, we examined the system by electrospray-ionization mass spectrometry (ESI-MS) under nondenaturing conditions. By careful instrumental optimization, use of near-neutral spraying buffer, and low desolvation gas temperature (30 °C), it is possible to observe a variety of noncovalently bound protein – ligand complexes in the gas phase.[8] Examples of bound ligands seen using this method include enzyme cofactors,[9] substrates,[10] and inhibitors.[11]

We postulated that it should be possible to extend this approach to the BmPBP – bombykol system, and thereby observe the noncovalent complex in the gas phase. Figure 1 shows the (transformed) native ESI mass spectrum of recombinant BmPBP alone, and the spectrum after exposure of BmPBP to bombykol (20 molar equivalents, overnight).[12] The largest peak in the upper trace (Figure 1a) displayed a mass of 15878 Da, a value identical to the theoretical mass deduced from the amino acid sequence of BmPBP, and to the measured mass using standard (denaturing) ESI-MS conditions (spraying solvent: water/acetonitrile (80/20) with 0.1% formic acid; data not shown). A second significant peak, at 16160 Da, only appeared under nondenaturing conditions and, therefore, was attributed to a noncovalent adduct of the BmPBP with an unknown contaminant of mass 282 Da. The impurity was probably introduced during expression, or purification, of the recombinant protein. It is interesting to note that oleic acid has a mass of 282 Da, and treatment of the PBP with an excess of this common lipid did lead to a significant increase in the 16160 Da peak. To date, however, we have not been able to confirm oleic acid as the contaminant.

When BmPBP was incubated with bombykol, a third ion was observed in the ESI mass spectrum (Figure 1b). In addition to the masses at 15878 (BmPBP) and 16160 Da (unknown BmPBP adduct), a large peak at 16116 Da was visible. This species was reproducibly 238 Da (= the molecular mass of bombykol) larger than the mass of BmPBP and was identified as the noncovalent BmPBP – bombykol complex [BmPBP+bombykol]. Lower molar excesses of bombykol produced a similar result (minimum excess measured was 5-fold).

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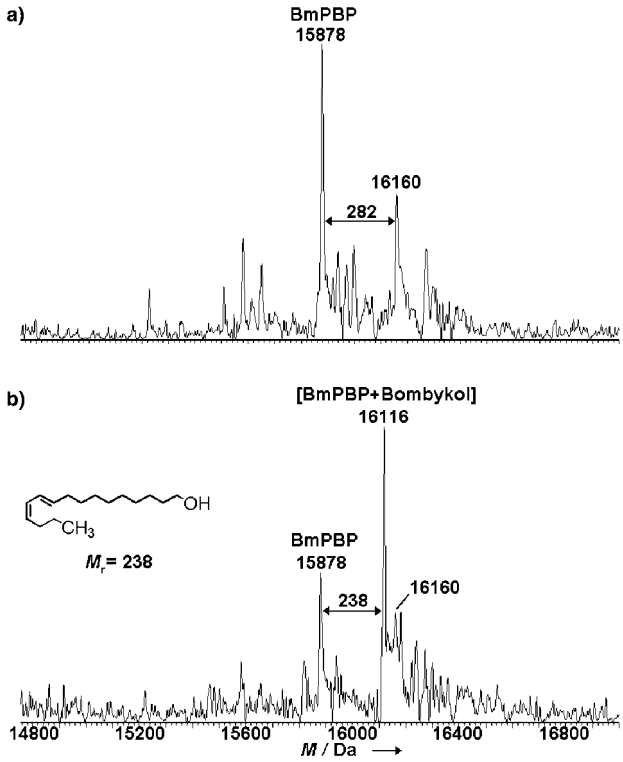

Figure 1. Transformed ESI mass spectrum of: a) BmPBP alone and b) BmPBP after exposure to bombykol. Data clearly show the presence of a [BmPBP+bombykol] complex at 16116 Da.[12]

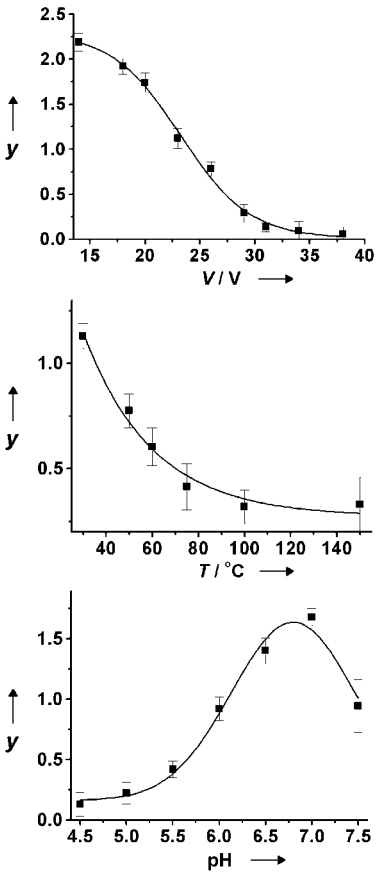

Figure 2. Effect of electrospray source parameters (cone voltage *V*, drying gas temperature *T*, and the pH value of the spraying buffer) on the stability of the [BmPBP+bombykol] complex (*y*-axis shows ratio of [BmPBP+bombykol] complex to free BmPBP).[12] Each data point is the mean value from five repeat injections (standard errors are indicated by the bars).

Figure 2 shows the result of varying the electrospray cone voltage *V*, the drying gas temperature *T*, and the pH value of the spraying buffer on the ratio of [BmPBP+bombykol] complex to free BmPBP. The electrospray sample cone voltage, which directly controls the acceleration of ions through the atmospheric pressure source into the high vacuum of the mass spectrometer, was found to be a critical parameter for observation of the noncovalent complex. Due to the effect of atmospheric-pressure collision processes, very little [BmPBP+bombykol] complex was observed at a cone voltage above 30 V. The temperature of the drying gas (nitrogen), used to evaporate the electrospray solvent droplets, also had an effect on the stability of the complex. Temperatures above 50 °C resulted in a rapid drop in the ratio of bound to free BmPBP, presumably caused by thermal denaturation of the protein. The spraying-buffer pH value exhibited a distinct optimum between pH 6.5 and 7.0, and the dramatic decrease in intact [BmPBP+bombykol] complex below pH 6.0 is consistent with the observed loss of the binding protein's rigid tertiary structure at low pH values.[13]

One potentially very useful application of this work is in studying the specificity of pheromone (or general odorant) binding proteins. Figure 3 shows that BmPBP does bind ligands that are structurally related to bombykol, such as (11*E*)- and (11*Z*)-hexadec-11-en-1-ol, but no evidence of a complex with the structurally unrelated alcohol 3-phenylpropan-1-ol was seen.[14] In addition, not only was it possible to distinguish between the ligand binding efficiencies of the monoene alcohols and bombykol, but even the geometric isomers of 11-hexadecenol gave significantly different

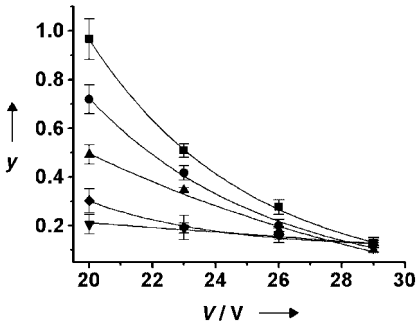

Figure 3. Comparison of BmPBP binding efficiencies with a range of ligands: ■ = bombykol, ● = (11*E*)-hexadec-11-en-1-ol, ▲ = (11*Z*)-hexadec-11-en-1-ol, ◆ = control, ▼ = 3-phenylpropan-1-ol.[14] The *y*-axis shows the ratio of [BmPBP+bombykol] complex to free BmPBP, *V* = cone voltage. Each data point is the mean value from 6–8 repeat injections (standard errors are indicated by the bars).

[BmPBP+ligand]:BmPBP ratios. Figure 3 illustrates that the 11*E* monoene appeared to bind better to the BmPBP than the 11*Z* form. Intriguingly, X-ray crystal structure data show that the C11–C12 bond of bound bombykol adopts a *transoid* conformation.[7]

The nondenaturing ESI-MS technique is a potentially powerful tool for assessment of the specificity of interactions between odorant molecules and olfactory binding proteins.

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Plans exist to extend this methodology to develop a competitive binding assay.

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[12] Mass spectra were recorded on a Micromass Quattro II mass spectrometer (Micromass, Manchester, UK) fitted with a Z-Spray electrospray source. NH_4OAc (2.5 mM, pH 7.0 unless otherwise stated) was used as a spraying buffer at a flow rate of 5 μL min^{-1}. The BmPBP solution (5 mg mL^{-1}, 2 μL, in 2.5 mM NH_4OAc, pH 7.0) was introduced into the flow using an injection valve. The cone voltage was 20 V and the drying gas temperature was 30 °C, unless otherwise stated. Recombinant BmPBP was produced and purified as previously described, except the hydroxylapatite column step was replaced by dialysis against 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.0) with a Slide-A-lyzer cassette (Pierce, Rockford, IL, USA).[5, 13] BmPBP was concentrated and transferred into NH_4OAc buffer (see above) with a Vivaspin concentrator (molecular weight cut-off 5000 Da; Vivascience, Lincoln, UK). Data was analyzed with MassLynx 3.1 (Micromass) software which had MaxEnt (maximum entropy based) software embedded.

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[14] The BmPBP solution[12] was incubated with a 50-fold molar excess of each ligand overnight at 4 °C. Alcohols were added to buffered BmPBP as 0.6 M ethanolic solutions. The control was BmPBP without added ligand. The nonzero values for the control result from baseline noise.

Chemo-, Regio-, and Stereoselective Cyclization of 1,3-Bis(trimethylsilyloxy)-1,3-butadienes with Functionalized Epoxides**

Peter Langer* and Tobias Eckardt

Domino reactions are of interest in organic chemistry since they enable the rapid assembly of complex products in a one-pot process.[1] Despite the simplicity of the idea, only few reactions of 1,3-dianions and 1,3-dianion equivalents with 1,2-dielectrophiles have been reported so far.[2] Several drawbacks hinder these reactions: on the one hand, dianions are highly reactive compounds that can react both as a nucleophile and a base; on the other hand, 1,2-dielectrophiles often represent rather labile compounds, which can undergo a series of side reactions (formation of open-chain 2:1 products, single-electron transfer (SET) reactions, elimination, polymerization, decomposition, fragmentation). In the course of work on the development of domino reactions of dianions and dianion equivalents,[3] we recently developed the first cyclization reaction of dilithiated 1,3-dicarbonyl compounds with oxalic acid dielectrophiles.[4] These reactions provide an efficient, regio- and stereoselective route to the pharmacologically important class of γ-alkylidenebutenolides.

Herein, we report, to our knowledge, the first Lewis acid mediated cyclizations of 1,3-bis(trimethylsilyloxy)-1,3-butadienes, electroneutral equivalents of 1,3-dicarbonyl dianions,[5] with epoxides. These reactions allow, for the first time, a highly efficient and chemoselective synthesis of 2-alkylidenetetrahydrofurans with a great variety of substitution patterns and functional groups.[6, 7] The cyclizations not only proceed with very good chemo-, but also with very good regio- and stereoselectivities. The products are useful precursors for the synthesis of pharmacologically relevant tetrahydrofuran derivatives and natural products.[8] The preparative usefulness of the new cyclization reaction was demonstrated by the synthesis of methyl nonactate, a known precursor to the natural product nonactin.

Our first attempts to induce a cyclization reaction of propenoxide **2a** with 1,3-bis(trimethylsilyloxy)-1,3-butadiene **1a**, which was prepared in two steps from ethyl acetoacetate,[5c] were unsuccessful (Scheme 1, Table 1). The use of $BF_3 \cdot OEt_2$ as the Lewis acid resulted in formation of a complex reaction mixture. Only starting materials were isolated when trimethylsilyl trifluoromethanesulfonate (Me_3SiOTf) was employed. Equally disappointing results were obtained when the reaction was carried out at 20 °C in the presence of $ZnCl_2$. A complex mixture was obtained when the reaction was carried out at 0 → 20 °C using $TiCl_4$ as the Lewis acid (Table 1, entry 4).

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