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Amino-Terminal Amino Acid Sequence of the Silkworm Prothoracicotropic Hormone: Homology with Insulin

Abstract. *Three molecular forms of prothoracicotropic hormone were isolated from the head of the adult silkworm,* Bombyx mori, *and the amino acid sequence of 19 amino acid residues in the amino terminus of these prothoracicotropic hormones was determined. These residues exhibit significant homology with insulin and insulin-like growth factors.*

Kopec (*1*) first described a factor from the brain of the gypsy moth, *Lymantria dispar,* that causes ecdysis and metamorphosis. Subsequent studies have shown that the brain secretes prothoracicotropic hormone (PTTH), which acts on the prothoracic glands to stimulate release of ecdysone (*2*). The head of the adult silkworm, *Bombyx mori,* contains two kinds of PTTH's with different molecular weights, previously named PTTH-S and PTTH-B according to species specificity (*3*) and now called 4K PTTH (molecular weight ~4400) and 22K PTTH (molecular weight ~22,000). The 4K PTTH is further composed of heterogeneous molecular species, one of which, designated 4K PTTH-I, has been isolated and partially characterized (*4*). We report here the isolation of two new 4K PTTH's, designated 4K PTTH-II and -III, and the NH_2-terminal amino acid sequences of 4K PTTH-I, -II, and -III.

The 4K PTTH-I (50 μg) was isolated from 648,000 heads of male adult *Bombyx* (4.86 kg) by a 15-step purification procedure involving heat-treatment, fractional precipitations, gel filtrations, ion-exchange chromatographies, and high-performance liquid chromatography (HPLC) (*4, 5*). The 4K PTTH's contain one or more disulfide bonds as the biological activity of the crude preparation (involving 4K PTTH-I, -II, and -III) was lost upon treatment with mercaptoethanol or dithiothreitol (*4, 6, 7*). Amino acid composition (molar ratios) of 4K PTTH-I is Asx(4), Thr(3), Ser(1), Glx(4), Pro(1), Gly(4), Ala(3), Cys(4), Val(4-5), Leu(5-6), Phe(2), Tyr(1-2), His(1), and Arg(3). Glycine was determined to be its NH_2-terminal residue by identification of the dansyl derivative (*4*). Injection of 0.1 ng of 4K PTTH-I induced adult development in a pupa of *Samia cynthia ricini* from which the brain had been removed (*8*). At a concentration of $1 \times 10^{-11} M$ (*4*), it could also activate a *Samia* prothoracic gland cultured in vitro.

The 4K PTTH-II and -III were separated from 4K PTTH-I in the 13th step of the procedure by ion-exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia) followed by elution with a gradient of 0.1 to 0.5*M* sodium chloride (*4, 5*). The 4K PTTH-I, -II, and -III were eluted from this column at 0.28, 0.30, and 0.33*M* sodium chloride, respectively. As in the case of 4K PTTH-I, reversed-phase HPLC on Develosil ODS-5 (Nomura-Kagaku) led to the isolation of 4K PTTH-II (36 μg) and -III (63 μg), which showed activity in a *Samia* pupa from which the brain had been removed at doses of 0.4 and 0.1 ng, respectively (Table 1).

The NH_2-terminal amino acid sequences of 4K PTTH-I, -II, and -III were determined by Edman degradation with the use of an Applied Biosystem model 470A gas phase sequencer. The phenylthiohydantoin (PTH) derivatives of the amino acids obtained at each cycle of the Edman degradation were identified by reversed-phase HPLC on an Ultrasphere ODS (Altex) column with a gradient of acetonitrile (10 to 50 percent) in 10 m*M* sodium acetate buffer (*p*H 4.5). Analyses were performed with 2.0 nmol each of 4K PTTH-I, -II, and -III. PTH–amino acids from cycles 1 to 19 were unambiguously determined by this method, although PTH–amino acids were not distinctly identified at cycles 6, 7, and 11 for 4K PTTH-I and -II, and at cycles 6, 7,

Table 1. Isolation of 4K PTTH-I, -II, and -III.

Purification step	Weight (mg)	Total activity* (10^3 *Samia* units)	Specific activity (nanograms per *Samia* unit)
Saline extract	$1{,}108 \times 10^3$	6,480	171,000
Crude 4K PTTH	19,400	6,480	3,000
Highly purified 4K PTTH	37.6	6,480	5.8
HPLC			
4K PTTH-I	0.050	520	0.1
4K PTTH-II	0.036	90	0.4
4K PTTH-III	0.063	630	0.1

*Bioassay. The samples [dissolved in 0.1*M* tris-HCl (*p*H 7.8) or 0.2*M* ammonium acetate] were injected routinely into five pupae of the saturniid moth *Samia cynthia ricini* that had aged 3 to 8 months after removal of the brain. When the sample possessed PTTH activity, the injected pupae underwent wing apolysis in 4 to 7 days. The activity is expressed in terms of the *Samia* unit, which is the minimum amount causing adult development in an assay pupa (*8*).

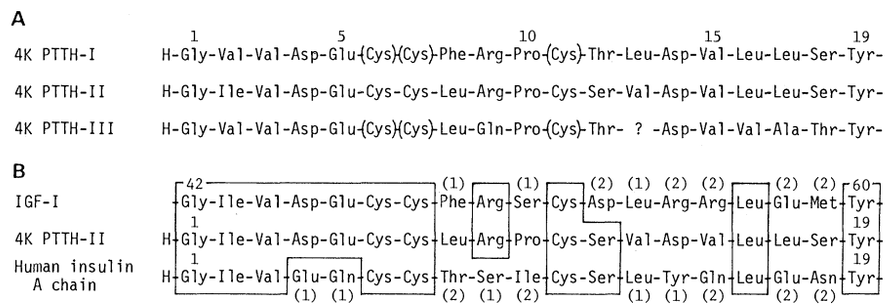

Fig. 1. (A) Amino-terminal amino acid sequence of 4K PTTH-I, -II, and -III. Cysteine residues at positions 6, 7, and 11 in 4K PTTH-I and -III have not yet been identified fully. (B) Homology between 4K PTTH-II and insulin-like growth factor I (IGF-I) and human insulin A chain. The boxed sections indicate identical amino acids. Numbers in parentheses are the minimum number of nucleotide base changes required to generate the amino acid substitution from that in 4K PTTH-II.

11, and 13 for -III. To establish the positions of cysteine residues, 4K PTTH-II was treated with dithiothreitol and then with iodoacetamide to generate the *S*-carboxamide methyl (CAM) derivative, which was subjected to Edman degradation. The PTH derivative of CAM-Cys was identified at cycles 6, 7, and 11, indicating the presence of Cys at positions 6, 7, and 11 of 4K PTTH-II. This suggests that 4K PTTH-I and -III also have Cys at the equivalent positions (Fig. 1A).

Almost half of the amino acids in the NH_2-terminal sequences are common to the three peptides. Amino acid substitution apparently occurs in such a way that the hydrophilic or hydrophobic nature of the amino acid residues is retained at their respective positions; Val is replaced by Ile, Phe by Leu, Arg by Gln, Thr by Ser, Leu by Val, and Leu by Ala. All the substitutions described above, except for that of Leu by Ala at position 17, would be possible by single nucleotide changes. Because all three hormones have approximately the same level of activity, we conclude that 4K PTTH's can tolerate substitutions at these positions and maintain biological activity. Replacement of Arg by Gln at position 8 in 4K PTTH-III may account for its being more acidic than 4K PTTH-I and -II. It is yet unknown whether a single silkworm has all three molecular species.

Molecules have been purified from the tobacco hornworm, *Manduca sexta* (*9*), and the blow fly *Calliphora vomitoria* (*10*), which are homologous in amino acid composition to vertebrate insulin, although their primary structures have not been proposed. It is of great interest that 4K PTTH's contain regions homologous with insulin A chain (*11*), insulin-like growth factors (IGF) (*12*), and a polypeptide with multiplication-stimulating activity (MSA) (*13*). Figure 1B shows the sequences of IGF-I (from positions 42 to 60), 4K PTTH-II (from positions 1 to 19), and human insulin A chain (from positions 1 to 19). Approximately half of the 4K PTTH-II sequence is identical to the IGF-I and human insulin A chain sequences. Cod and toadfish insulin A chain, not shown here, are even more homologous to 4K-PTTH-II (58 percent) (*11*). The differences in insulin A chain that occur among vertebrates (including mammals, fish, and birds) are located at positions 4, 8 to 10, 12 to 15, 17, and 18 (*11*). The 4K PTTH-II differs from human insulin A chain in its sequence at all of these positions except for position 12. This is accompanied by an additional substitution at position 5. Porcine insulin (Sigma) failed to show PTTH activity at a dose of 1 μg, which is about 10^4 times the minimal active dose of 4K PTTH's. The 4K PTTH's failed to bind to guinea pig antibody to porcine insulin (Miles) as measured by radioimmunoassay (*14*). These data suggest the existence of a common ancestral peptide molecule that evolved into peptides with different functions in insects and vertebrates.

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5. Isolation of 4K PTTH species. *Bombyx* adult heads, homogenized successively in acetone and 80 percent aqueous ethanol, were extracted three times with 2 percent aqueous sodium chloride. The extract was heated (100°C) and centrifuged to remove the precipitate. This was followed by ammonium sulfate precipitation and two extractions with acetone. The precipitate was then solubilized in water, mixed with nine volumes of a saturated aqueous solution of picric acid, and the precipitate appearing after centrifugation was dissolved in 0.1*M* tris-HCl (*p*H 7.8). After a final extraction with acetone, the pale brown precipitate, designated as "crude 4K PTTH," was dissolved in 0.1*M* tris-HCl (*p*H 7.8), subjected to gel filtration on Sephadex G-50 (fine; 6 by 67 cm), and eluted with 0.2*M* ammonium acetate (250 ml/hour). The pooled active fractions were then applied to a DEAE-Sepharose CL-6B column (2.6 by 40 cm) that had been equilibrated with 0.2*M* ammonium acetate. The column was washed with 0.4*M* ammonium acetate and eluted with 1*M* acetic acid (20 ml/hour). The eluate was then charged onto an SP-Sephadex C-25 column (1.4 by 28 cm) that had been pretreated with 0.1*M* ammonium acetate buffer (*p*H 4.2). The pooled active fractions were lyophilized, dissolved in 0.1*M* tris HCl (*p*H 7.8), and applied to a Sephadex G-50 (superfine) column (1.8 by 170 cm), which was eluted with 0.2*M* ammonium acetate (8 ml/hour). The active fractions were combined (designated as "highly purified 4K PTTH") and applied to a DEAE-Sepharose CL-6B column (1 by 54 cm) equilibrated with 0.05*M* tris-HCl containing 0.1*M* sodium chloride. The column was washed with the same solution and then eluted with a gradient of 0.1 to 0.5*M* sodium chloride in 0.05*M* tris-HCl (*p*H 7.8) (8 ml/hour), while fractions (3.5 ml) were collected. The isolates 4K PTTH-I, -II, and -III were recovered from tubes 71 to 73, 74 to 76, and 83 to 89, respectively.
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Voltage-Dependent Calcium Channels in Glial Cells

Abstract. *The electrophysiological properties of glial cells were examined in primary culture in the presence of tetraethylammonium and Ba^{2+}, a treatment that reduces K^+ permeability of the membrane and enhances currents through voltage-dependent Ca^{2+} channels. Under these conditions, glial cells showed both spontaneous action potentials and action potentials evoked by the injections of current. These responses appear to represent entry of Ba^{2+} through Ca^{2+} channels because they were resistant to tetrodotoxin but were blocked by Mn^{2+} or Cd^{2+}.*

Glial cells are considered the ubiquitous yet silent partners of neurons. They appear to play a complex supportive role in nervous tissue (*1–3*). The syncytium formed by glial cells is believed to be important in controlling the extracellular milieu surrounding nerve cells by buffering potassium (*4*), by uptake of neurotransmitters (*2*) and transport of nutrients from the blood (*5*), and by providing mechanical support for the neuronal matrix. Glial membranes are also thought to be exclusively permeable to K^+ (*3*), and active voltage-dependent responses have not been reported. However, in their original work on the properties of glial cells, Kuffler and Potter (*6*) noted that the "steady-state resistance in some glial cells dropped when depolarizations were larger than 20–30 mV." Other workers have reported that Ca^{2+} influx is increased with high extracellular K^+ (*7*). The pronounced K^+ permeability of glial membranes could shunt and mask any voltage-dependent responses. Moreover, in most studies in which the permeability of glial membranes to ions other than K^+ was examined (*8*), the stable membrane potential was measured in response to changes in extracellular K^+.