Biosynthesis of Adipokinetic Hormones (AKHs): Further Characterization of Precursors and Identification of Novel Products of Processing

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Two adipokinetic hormones (AKH I: pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH₂, and AKH II: pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH₂) are synthesized by the neurosecretory cells of the corpora cardiaca (CC) of the locust *Schistocerca gregaria*. Both AKHs are released into the blood during flight and serve to regulate lipid metabolism and other physiological processes involved in flight. By *in vitro* "pulse-chase" experiments we show that 2 precursors (P1 and P2) are involved in AKH biosynthesis. These are about 8.4 kDa polypeptides which are AKH immunoreactive but also contain an amino acid (tyrosine) not present in the AKH peptides. By following the fate of ¹⁴C-tyrosine incorporated into P1 and P2, we have identified 2 novel products of precursor processing. These are called AKH-Precursor Related Peptides or APRP 1 and APRP 2. Size exclusion chromatography shows that the APRPs are about 6.5 kDa large and therefore represent major fragments of the precursors.

The optical density peaks corresponding to P1, P2, APRP 1, and APRP 2 on a reverse-phase chromatogram are identified. The precursors being metabolic intermediates are represented by minor optical density peaks that disappear when *de novo* protein synthesis is blocked by cycloheximide. In contrast, the APRPs are represented by major optical density peaks consistent with their being accumulating end products of AKH precursor processing. The function of the APRPs is as yet unknown. They are, however, co-synthesized and also co-released with the AKHs, and may therefore also have hormonal functions related to flight.

It is now well established that the precursor for a peptide transmitter or hormone may include more than one bioactive amino acid sequence (for reviews, see Lynch and Snyder, 1986; Eipper et al., 1986). During enzymatic processing of the precursor, different bioactive sequences can therefore be liberated and made available for release. There are few intact tissue systems in which this aspect of peptide biosynthesis can conveniently be studied. One such system is represented by the bag cell cluster of *Aplysia*. These cells produce the precursor of egg laying hormone (ELH), which upon processing generates ELH and additional peptides that appear to be necessary for the expression of the complete egg laying behavior (Scheller et al., 1983; Mayeri et al., 1985;

Sigvardt et al., 1986). We have previously described (Hekimi and O'Shea, 1987) another tissue that allows us to study the biosynthesis and identify 2 precursors (P1 and P2) of 2 related insect neuropeptides (the adipokinetic hormones, or AKHs). Here, we identify 2 novel peptides generated from these precursors and characterize them as major fragments of their respective precursors.

Our studies were initiated with the adipokinetic hormones (AKH I and AKH II) synthesized by the neurosecretory cells of the glandular lobes of the corpora cardiaca (CC) of the locust *Schistocerca gregaria*. The CC are paired neurosecretory glands located in the head in close proximity to the brain, by which they are innervated. In the adult locust the AKH peptides are released just after the initiation of flight. This results in a mobilization of lipid metabolism, which provides the energy for migratory flight. The amino acid sequence of AKH I is pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH₂ (Stone et al., 1976) and for AKH II pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH₂ (Siegert et al., 1985). These hormones are major products of the neurosecretory cells of the locust CC. The CC (about 0.1 mg wet weight) contain as much as 1 nmol AKH I and 0.2 nmol AKH II. This remarkable tissue therefore provides an unusually good system for the analysis of the synthesis of neuropeptides.

Synthesis of AKH I and AKH II can be studied by providing radiolabeled amino acids to CC maintained in short-term tissue culture (Hekimi and O'Shea, 1987). By following the incorporation of ³H-tryptophan, we showed that label is first incorporated in 2 polypeptides called P1 and P2 prior to its appearance in AKH I and II. On the basis of this observation and by showing that P1 and P2 are AKH-immunoreactive, we identified them as precursors of the AKHs. In addition, we found that biosynthetically labeled P1 and P2 and their AKH immunoreactivity co-migrated with 2 major optical density (OD) peaks detected using reverse-phase chromatography. This led us to believe that the 2 OD peaks were due to the presence of relatively large amounts of the AKH precursors. In this respect, we were wrong. We show here that P1 and P2 and their AKH immunoreactivity can be separated from the major OD peaks. Relatively small optical density peaks are now assigned to the precursors, consistent with P1 and P2 being nonaccumulating intermediates of AKH biosynthesis. We show now that the large OD peaks are produced by 2 major fragments of the precursors, generated during processing. They accumulate in the CC in amounts comparable to the amounts of the AKHs. We have named these newly identified products *Adipokinetic hormone-Precursor Related Peptides*, or APRPs. Although derived from the AKH precursors, the APRPs are themselves neither AKH-immuno-

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Figure 1. Reverse-phase chromatogram of 30 CC showing double-labeled (³H-tryptophan and ¹⁴C-tyrosine) P1 and P2 (upper panel) and the AKH-like immunoreactivity associated with each fraction of the eluant measured by RIA (lower panel). Note that the peak fraction of the 2 isotopes coelutes for both P1 and P2, and, moreover, the shapes of the peaks defined by the 2 isotopes are the same. The peaks of double-labeled precursors also coelute with the AKH-like immunoreactivity. In this experiment each fraction was split in 2 parts, one being subjected to RIA corresponding to 25 CC and the other was monitored for ³H- and ¹⁴C-decays.

reactive nor do they contain tryptophan. While their function is unknown, their synthesis is coupled to that of the AKHs, and we show that they are released with them. The APRPs might therefore be hormones with activities that complement those of the AKHs (see Discussion).

Materials and Methods

Animals. Both male and female adult specimens of the locust *Schistocerca gregaria* were used in our experiments. They were reared in our own laboratory culture. Female New Zealand White Rabbits produced the antiserum to a synthetic analog of AKH I.

Preparation of AKH antiserum and radioimmunoassay procedures. To generate an antiserum to the precursor form of AKH, an analog of the sequence Lys-Tyr-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-OH was designed and synthesized (Peninsula Laboratories). Details of methods used to generate antisera and those used in the radioimmunoassay (RIA) were given in Hekimi and O'Shea (1987), in which the serum is named IIIa. Here, the tracer for the radioimmunoassay (RIA) was a synthetic analog of the sequence Tyr-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH₂ (Peninsula Laboratories). This peptide was radiolabeled on the tyrosine residue with ¹²⁵I. Activated charcoal was used to precipitate unbound peptide after serum IIIa (diluted 20-fold) was incubated with the tracer and samples. The ability of material from HPLC fractions to displace bound counts was determined.

Organ (CC) culture. Details of *in vitro* incubation methods were given in Hekimi and O'Shea (1987). In order to monitor synthesis of the precursors and their products 10 μ Ci of [³H]side chain 2,3] L-tryptophan (60 Ci/mM) or 10 μ Ci of L-(U-¹⁴C) tyrosine (500 mCi/mM) or both was added to the incubation saline. Following varying durations of incubation (see Results) with labeled amino acids, the CC were either washed in saline and extracted (see below) or transferred to a large volume (5 ml) of "translation-block" medium. The translation-block medium consisted of saline containing 1 μ g/ml cycloheximide.

Extraction, identification, and purification procedures. Corpora cardiaca were homogenized in 100 μ l of 0.1% trifluoroacetic acid (TFA)

at room temperature with a Branson microprobe ultrasound cell disruptor. The crude TFA-soluble supernatant was removed, frozen immediately, and subjected to size-exclusion and/or reverse-phase chromatography.

For size fractionation, a 60 cm TSK 2000 SW gel-permeation column (LKB), eluted at 0.5 ml/min with 0.1% TFA containing 15% (vol/vol) acetonitrile was used. The exclusion size of this column is about 50 kDa. Acetonitrile was used to prevent hydrophobic interactions, which can result in anomalous retentions of some peptides and tryptophan. The TSK 2000 column was calibrated according to molecular weight using the following standards: myoglobin (fragment) 14.4 kDa; myoglobin (fragment) 8.2 kDa; myoglobin (fragment) 6.2 kDa; a synthetic 28 residue peptide of 3.2 kDa. Reverse-phase separations of AKH I, AKH II, APRP 1, APRP 2, P1, and P2 were achieved using a C₈ wide-pore column (Aquapore RP-300; 7 μ m, Pierce Chemical Company). The column was eluted at 1.5 ml/min with 0.1% TFA and a linear acetonitrile gradient (from 25 to 45% in 10 min). Retention times of synthetic AKH I or II (Peninsula Laboratories) could not be distinguished from those of native unlabeled and native biosynthetically labeled AKH I or II.

Three types of detection systems were used to identify the eluting compounds. On-line detection was provided by a Waters 440 fixed-wavelength absorbance detector set at 214 nm, which gave an estimate of the relative abundance of peptide bonds. In one experiment (Fig. 1), compounds were detected by RIA and scintillation counting after fractionation of the eluant. For detection of the AKH immunoreactivity, HPLC fractions were evaporated to dryness using a Savant Speed-Vac evaporator and subjected to RIA (see above). For calculation of the isotopic content of HPLC fractions, ³H and ¹⁴C β -emissions were distinguished by their respective energies.

Potassium-stimulated calcium-dependent release. The ability of CC to release the APRPs was investigated by superfusing the glands with physiological saline and with salines of altered ionic composition. In order to concentrate released hydrophobic compounds, the glands were placed in a small chamber (Swinny Filter Holder, Millipore) attached above a cartridge containing reverse-phase packing (C₁₈ SepPak, Waters Associate). The superfusate was added above the chamber, and a peri-

Figure 2. Pulse-translation block experiment using reverse-phase chromatography of CC after incubation with ^{14}C -tyrosine and ^3H -tryptophan for 1.5 hr followed by 24 hr block. *Left panel.* Chromatogram just after the incubation period. Both isotopes are incorporated into both P1 and P2 as indicated by the co-migration of the ^3H (above) and ^{14}C (below). *Right panel.* Chromatogram of CC incubated in cycloheximide for 24 hr after the 1.5 hr pulse labeling. Label P1 and P2 is now transferred to the AKHs (^3H) and the APRPs (^{14}C).

staltic pump was used to move it through the chamber and SepPak. Further details of this method for monitoring peptide release have been described (Adams and O'Shea, 1983; O'Shea et al., 1984). Fifty CC were placed in the chamber and superfused for 1 hr each in succession with 3 types of saline. The first was physiological saline, the second contained no calcium but had elevated potassium concentration (50 mM), and the third had elevated potassium concentration with normal (5 mM) calcium. For each saline condition a new activated SepPak was attached to the chamber. The SepPaks were flushed with distilled water and the trapped peptides eluted in 5 ml of methanol. Following evaporation of the methanol, the samples were analyzed by reverse-phase chromatography for the presence of APRPs.

Results

Identification of 2 AKH-precursor related peptides (APRPs)

In vitro incubation of CC with ^3H -tryptophan results in the synthesis of 4 major tryptophan-containing peptides. These are AKH I, AKH II, and their precursors P1 and P2 (Hekimi and O'Shea, 1987). In our previous publication, the AKH-precursor status of P1 and P2 was established by the following criteria. Both P1 and P2 are larger than the AKH peptides and both are synthesized prior to them. By performing pulse-chase or pulse-

translation block experiments, tryptophan incorporated in P1 and P2 can be recovered in AKH I and II. Finally, both P1 and P2 are AKH-immunoreactive, indicating that they contain amino acid sequences related to AKH.

In order to investigate whether P1 and P2 could contain peptides other than AKH, we cultured CC in the presence of ^3H -tryptophan, an amino acid present in the AKHs and ^{14}C -tyrosine, an amino acid not present in the AKHs. Figure 1 shows a C_8 reverse-phase chromatogram of an extract of CC that was incubated for 1.5 hr with both ^3H -tryptophan and ^{14}C -tyrosine. Two peaks labeled with both isotopes (^3H and ^{14}C) can be seen. These were identified as P1 and P2 by several criteria. For example, they occur with the expected retention time and amplitudes (the peak of P1 predominates) and both are AKH-immunoreactive (see lower part of Fig. 1). Note that the ^3H and ^{14}C peaks are virtually superposed, indicating that tyrosine, in addition to tryptophan (as shown previously), is incorporated in the AKH precursors. Thus, the processing of P1 and P2 might result in the production of tyrosine-containing peptides, in addition to the AKHs.

Figure 3. Size characterization by TSK 2000 size exclusion chromatography of reverse-phase purified P1, P2, APRP 1, and APRP 2. The upper panel shows the OD at 214 nm associated with the chromatographic profiles of the labeled compounds. Two chromatographic runs were performed, one included purified P1 plus APRP 1 and the other P2 plus APRP 2 (equal CC equivalents for each). Note that the OD peaks co-migrate with the peaks of labeled APRPs and not with the labeled Ps.

That this is the case is shown in Figure 2, in which CC pulse-labeled with ^3H -tryptophan and ^{14}C -tyrosine are placed in a "translation-block" medium containing cycloheximide. Note that during the brief incubation (1.5 hr) no radiolabeled AKH is found. Following a 24 hr period of translation block, the double-labeled precursors have disappeared and the tritium tryptophan is chased into the 2 AKH peptides, as expected. Transfer of ^3H -tryptophan to the 2 AKHs is accompanied by the transfer of ^{14}C -tyrosine into 2 new radiolabeled compounds (APRP 1 and 2), migrating close to the double-labeled precursors (now absent).

The fact that APRP 1 and 2 migrate on a reverse-phase column very close to the P1 and P2 precursors suggests they are similar in hydrophobicity to their parent compounds. This could

Figure 4. Pulse-translation block time-course experiment showing size-exclusion chromatograms of CC after incubation for 1.5 hr with ^3H -tryptophan and ^{14}C -tyrosine. Immediately following the incubation CC were extracted and chromatographed (top) and then after 4, 12, and 24 hr in the presence of cycloheximide. The figure shows a progressive transfer of ^{14}C -tyrosine from the larger Ps to the smaller APRPs during translation block. As the double-labeled peak (P1 and P2) disappears the ^3H -labeled tryptophan in this peak is transferred to the AKH peptide (not included in these chromatograms) and the ^{14}C -tyrosine is transferred to the APRPs. Thus, the ^{14}C -tyrosine is shifted progressively to the right (smaller molecular weight).

arise if they represent major fragments of the precursors. Sizing of the APRPs confirms this. To investigate the size relationships between the newly identified APRPs and their precursors we first purified P1, P2, and APRP 1 and 2 by reverse-phase chromatography and then chromatographed them by size exclusion. This is shown in Figure 3. In this experiment CC were pulse labeled for 1.5 hr with either ^3H -tryptophan or ^{14}C -tyrosine. The tryptophan-labeled P1 and P2 were separated and purified by C_8 reverse-phase HPLC. The glands pulsed with ^{14}C -tyrosine,

Figure 5. Reverse-phase chromatogram of 3 adult CC (glandular lobes). Only 4 major optical density (214 nm) peaks are seen, AKH I and II and APRP 1 and APRP 2. The first peaks seen to the left represent the solvent front. The simplicity of the OD profile suggests that the 4 peaks seen represent the major stored products of the neurosecretory cells of the glandular lobe.

however, were transferred to the translation-block medium overnight to allow processing of the pulse-labeled P1 and P2 into the ^{14}C -labeled APRPs. These glands were also subjected to C_8 reverse-phase chromatography and the 2 ^{14}C -labeled products (APRP 1 and 2) collected separately. Thus, we produced purified ^3H -tryptophan P1 and P2 and purified ^{14}C -tyrosine APRP 1 and APRP 2.

These purified compounds were then applied to a size-exclusion column (TSK 2000) in the following way (Fig. 3). The ^3H -labeled P1 was mixed with ^{14}C -labeled APRP 1 and similarly labeled P2 was mixed with labeled APRP 2. The mixtures included the same tissue (CC) equivalent amounts of the 2 compounds (a P and an APRP). This allowed us to see the relative molecular weights of a P and an APRP simultaneously while monitoring the relative contribution made by each to the OD (a measure of relative amounts per gland). Three expectations were confirmed by this experiment. First, the APRPs are smaller than the Ps. Second, the APRPs are only slightly smaller than the Ps, indicating that they are major fragments of their precursors. Third, monitoring of the column eluant at 214 nm shows that the APRPs are present in the CC in much larger amounts than the Ps (see also Fig. 6). This is expected because precursors are metabolic intermediates but their products accumulate.

Estimates of sizes were derived from the TSK 2000 column calibrated using polypeptide standards spanning the range from 14.4 to 3.2 kDa (see Materials and Methods). This gave molecular weights of about 8.4 kDa for P1 and P2 and about 6.5 kDa for APRP 1 and APRP 2. Thus, the APRPs represent approximately 80% of the size of their precursors. The difference in size (about 2 kDa) between a P and an APRP allows the precursors to include no more than 2 copies of an AKH peptide (see Discussion).

The separation of the Ps from the APRPs achieved by the TSK 2000 column allowed us to follow the time dependence of the transfer of ^{14}C -tyrosine from the larger Ps into the smaller APRPs. We exploited the fact that Ps can be double-labeled (with ^{14}C -tyrosine and ^3H -tryptophan) and that the tyrosine is

transferred to the APRPs after processing. In this way we have been able to show the expected time-dependent transfer of label from precursors (P1 + P2) into the APRPs. This experiment is shown in Figure 4. The CC were first incubated for 1.5 hr in ^3H -tryptophan and ^{14}C -tyrosine, thus labeling P1 and P2. Immediately following this incubation, a broad double-labeled peak representing the sum of P1 and P2 is seen by size-exclusion chromatography (Fig. 4, upper panel). That this broad peak consists of P1 and P2 can be confirmed by collecting this material and rechromatographing it by C_8 reverse phase. Following the pulse labeling, CC were transferred into the translation-block medium and then sampled periodically. Figure 4 shows the results following periods of 4, 12, and 24 hr in cycloheximide. Since during these periods new translation but not processing is blocked, there is a progressive, time-dependent decline in the double-labeled P1 + P2 peak. The ^3H -tryptophan is transferred to the AKH I and II peptides (Hekimi and O'Shea, 1987; not shown in Fig. 4). The ^{14}C -tyrosine of the double-labeled peak is shifted slightly to the right, as tyrosine-labeled APRPs are synthesized. When chromatographed together, APRP 1 is not well resolved from APRP 2, resulting in the bilobed appearance of the APRP peak at the end of the 24 hr period in cycloheximide (Fig. 4, lower panel).

OD peaks assigned to Ps and APRPs

The results so far can be summarized by saying that 2 precursors P1 and P2 (about 8.4 kDa) are synthesized and metabolized and processed rapidly in the CC, producing 4 accumulating compounds, AKH I and II (1.1 and 0.8 kDa) and APRP 1 and 2 (each about 6.5 kDa). The APRPs therefore represent large fragments of the Ps, the difference in size being about 1 or 2 AKH peptides. Size considerations suggest that the AKHs and APRPs may be the only accumulating products of AKH-precursor processing in the CC. A reverse-phase chromatogram of a CC extract reflects this simplicity (Fig. 5) in which 4 major optical density peaks are seen. In Figure 5, the optical density peaks marked APRP 1 and APRP 2 co-migrate with the biosynthetically labeled products of P1 and P2 processing (see Fig. 2). Note that each APRP is preceded by a very minor optical density peak, barely visible in Figure 5 but more clearly shown in Figure 6. These peaks were not detected in our previous report (Hekimi and O'Shea, 1987) because the C_8 column (Watman Protesil 300 Å octyl) and conditions used in those experiments did not permit the separation of the smaller from the larger OD peaks. The chromatogram shown in Figure 6 is from a Pearce C_8 column using a gradient designed to optimize separation in this region (see Materials and Methods). We can now show that the ^3H -tryptophan-labeled P1 and P2 do not co-migrate with major OD peaks but with the 2 minor OD peaks. This was to be expected from the results shown in Figure 3 in which significant OD is associated with the APRPs and not the Ps.

If the 2 minor OD peaks co-migrating with biosynthetically labeled precursors are indeed due to the precursors, their amplitudes should be diminished when P1 and P2 are processed under conditions that prevent their continued synthesis. Such conditions are achieved by placing the glands in cycloheximide. So we performed the following experiment. *Corpora cardiaca* were first incubated in the presence of both ^{14}C -tyrosine and ^3H -tryptophan for 1.5 hr. During this brief period the P1 and P2 precursors become double-labeled, as shown previously. The biosynthetically labeled P1 and P2 co-migrate with the 2 minor OD peaks (Fig. 6, left panel). After pulse labeling some of the

Figure 6. A pulse-translation block experiment using reverse-phase chromatography. In the *left panel*, P1 and P2 were double-labeled with ³H-tryptophan and ¹⁴C-tyrosine for a 1.5 hr pulse incubation. Note that the double-labeled precursors do not co-migrate with the major OD peaks but with the minor ones shown above. After 24 hr in cycloheximide (*right panel*), the double-labeled precursors have disappeared and so have the small OD peaks co-migrating with them. The ¹⁴C-tyrosine peaks are now shifted to the right and each lies under a major OD peak. The *lower panel* shows that the small OD peaks associated with the P1 and P2 precursors are not diminished after 24 hr in physiological saline without cycloheximide. Note in *right panel* that only the P1 and P2 associated OD peaks are lost, the third minor peak eluting earlier being unchanged in all panels.

CC were transferred to the translation-block medium for 24 hr. Following this, ¹⁴C-labeled tyrosine no longer co-migrates with P1 and P2 but is shifted to the right to lie under the larger OD peaks produced by the APRPs (Fig. 6, right panel). This result was expected from previous experiments (Fig. 2). Note that the ³H-tryptophan is no longer present because it has been transferred to the AKHs (not shown in Fig. 6). The new finding shown here is that the disappearance of label from P1 and P2, indicating complete processing of available precursor pool, is accompanied by the disappearance of the small OD peaks. Thus, in conditions under which new precursor synthesis is prevented, the small OD peaks behave like the biosynthetically labeled precursor

peaks, that is, they are "chased out." In a control experiment, some of the glands that received the double-labeled pulse were washed and transferred to saline without cycloheximide for 24 hr. Under these conditions, we would not expect the steady-state levels of the precursors to change because P1 and P2 synthesis can continue. The OD associated with P1 and P2 therefore should not be diminished. This is confirmed in the lower panel of Figure 6.

Calcium-dependent release of the APRPs

The demonstration of co-synthesis of the APRPs and the AKH peptides suggests that the APRPs may be hormones. Questions

Figure 7. Calcium-dependent release of APRP 1 and APRP 2 from the CC activated by high (50 mM) potassium saline. The released peptides were analyzed by reverse-phase chromatography and comparison of release made by measuring optical density at 214 nm (relative scale: the OD associated with APRP 1 released under zero calcium is taken as unity). Background release in normal saline was subtracted from the release under the 2 conditions shown (high K⁺, zero added Ca²⁺, and high K⁺, 5 mM Ca²⁺).

of the biological function of the APRPs, however, are not yet resolved. Circumstantial evidence for a hormonal role, however, is provided by a demonstration that the APRPs are released from the CC in a voltage- and calcium-dependent manner. Figure 7 shows that both APRP 1 and APRP 2 are released from the CC by exposure to saline containing 50 mM potassium. The above-background (release in normal saline subtracted) release is approximately 5 times greater in the presence of 5 mM calcium. Note also that the 2 APRPs are released in approximately the same ratios as found in the CC. That is, about twice as much APRP 1 as APRP 2. Similar release data have been previously published for the AKHs and AKH-homologous peptides (O'Shea et al., 1984; Pannabecker and Orchard, 1987). Our own release experiments show that the AKHs are co-released with the APRPs (not shown).

Discussion

Our results provide evidence for the existence of 2 new peptides derived from the 2 precursors of the AKH peptides. That P1 and P2 are precursors of AKH I and AKH II is based on 2 types of evidence. First, pulse-chase experiments show that tryptophan incorporated into P1 and P2 is recovered in AKH I and AKH II after processing. Second, P1 and P2 cross-react with an anti-AKH serum. Together these observations strongly support the role of P1 and P2 as AKH precursors (Hekimi and O'Shea, 1987). The evidence that P1 and P2 are in addition the precursors of APRP 1 and APRP 2 is derived from a series of quantitative pulse-chase experiments. In summary, the P1 and P2 precursors incorporate tyrosine, an amino acid absent from

the AKHs. The incorporated tyrosine can be recovered in the 2 identified APRPs when translation, but not processing, is blocked. No other tyrosine-labeled compound besides P1 and P2 can account for the amount of label appearing in the APRPs following a pulse-chase experiment. Thus, P1 and P2, the AKH precursors, appear to be the only candidates for the precursors of the APRPs. The strategy summarized above for the discovery of novel products of hormone precursor processing is perhaps possible only with a highly homogenous cell population such as found in the glandular lobes of the CC.

In addition to providing evidence that the APRPs are derived from the AKH precursors, we also show that they are major fragments of their parent polypeptides. In fact, our size estimates show that P1 and P2 can each accommodate only 1 APRP and 1 but not more than 2 AKH sequences. The estimated size of P1 and P2 (8.4 kDa) can accommodate very little more. The issue of the APRP and AKH content of the precursors will be resolved unambiguously only by determination of the complete amino acid sequences of P1 and P2. Sequencing work on the Ps and APRPs is in progress (S. Hekimi and M. O'Shea, unpublished observations) and results confirm the predictions from the pulse-chase experiments reported here.

A reverse-phase chromatogram of extracts of the glandular cells of the CC shows only 4 major OD peaks. Two of these are AKH I and AKH II. The other 2 major OD peaks are the APRPs. Our present results show that 2 minor OD peaks are associated with P1 and P2. The amplitudes of these peaks reflect the steady-state levels of these precursors. Since precursors are not terminal products but metabolic intermediates, P1 and P2 do not accumulate and their steady-state levels would be expected to be low. In contrast, the 2 AKHs and the 2 APRPs are accumulating stored products of biosynthesis and are thus present in large amounts and generate the major OD peaks of the CC extract.

Several important questions remain to be addressed following our identification of APRPs in the locust CC. What, for example, are their functions? We know from other systems that peptide precursors often contain sequences of more than one type of biologically active peptide. Perhaps P1 and P2 are not exceptional in this respect, and the APRPs are peptide hormones with as yet undetermined biological activity. At present, the APRPs remain putative hormones as they were identified by a strategy in which biological activity was not used as a guide for purification. The AKHs and APRPs are however co-released by elevated potassium saline. Thus, the APRPs might be released at the initiation of flight behavior, as are the AKHs, and they may also be involved in regulating physiological functions important in that behavior.

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