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Identity of a second type of allatostatin from cockroach brains: An octadecapeptide amide with a tyrosine-rich address sequence

(insect/amino acid sequence/neuropeptide/juvenile hormone)

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ABSTRACT **An octadecapeptide that inhibits juvenile hormone synthesis has been isolated by HPLC from brain-retrocerebral complexes of the cockroach *Diploptera punctata*. The primary structure of this allatostatin has been elucidated by tandem mass spectrometry: Ala-Tyr-Ser-Tyr-Val-Ser-Glu-Tyr-Lys-Arg-Leu-Pro-Val-Tyr-Asn-Phe-Gly-Leu-NH_2 (ASB2). The amidated three-residue C terminus of this type B allatostatin is identical to that of four known type A allatostatins, and the preceding three residues show close structural homology. ASB2 has over twice the activity of the type A tridecapeptide Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH_2 (ASA1) in inhibiting juvenile hormone biosynthesis in corpora allata from females in early vitellogenesis (day 2), and its efficacy persists during pregnancy, but it is equally effective as ASA1 on glands from day-10 females (IC_{50} = 0.31 nM). The octadecapeptide is characterized by a potential dibasic cleavage site, Lys^9–Arg^{10}, the integrity of which is needed for high potency. The ASB2-(11–18)-octapeptide amide gives a full response at high concentrations at day 10 (IC_{50} = 48 nM), but the C-truncated (1–9)-, (1–11)-, and (1–17)-amide fragments of ASB2 are inactive. Thus, the endocrine message is located at the C terminus. N^{α}-acetylation of the N-truncated (9–18), (10–18), and (11–18) fragments of ASB2 increases activity relative to the nonacetylated peptides. The site of action of type A and type B allatostatins is located before mevalonate kinase in the biosynthetic pathway for juvenile hormone.**

Metamorphosis and reproduction are controlled in most species of insect by the juvenile hormones (JH), sesquiterpenoids produced by the corpora allata (CA). The activity of these endocrine glands is regulated by a variety of humoral and nervous factors originating, at least in part, in the insect brain (1). The chemical identity of these factors is still largely unknown, despite their appeal as lead compounds in the design of modern tools for insect pest management (2, 3). Allatotropin, a 13-residue peptide, purified from heads of pharate adult *Manduca sexta* was shown to stimulate JH synthesis by CA of this lepidopteran species (4). On the other hand, cockroach allatostatins are a family of basic neuropeptide amides that cause rapidly reversible inhibition of JH synthesis in isolated CA (5–8), through regulation of some, as yet unidentified, step(s) early in the biosynthetic pathway (6). They have been studied mostly for their action on glands from adult females in relation to the reproductive cycle (5–8) but are also reported to act on larval CA (5) and, hence, may also have a role in metamorphosis. Compounds with similar function probably exist in other insects (9, 10). The primary structures of an octapeptide amide and tridecapeptide amide from brains of *Diploptera punctata* were independently determined in two laboratories (5–7), and related nonapeptide and decapeptide amides have also been found (5). All four peptides exhibit C-terminal amidation, which is known to be essential for bioactivity (6); presumably this is derived from a glycine residue in the prohormone(s) (11). These type A allatostatins show a highly conserved C-terminal sequence (-Leu-Tyr-Xaa-Phe-Gly-Leu-NH_2), whereas the N termini show greater variation in composition and length. All four peptides exhibit some activity at concentrations <100 nM, but the tridecapeptide ASA1 has the highest activity in two routine bioassays (6, 7). This result is consistent with the N terminus acting as an address segment to promote high-affinity interaction (7).

Our procedure for the isolation of type A allatostatins from total extracts of brain–corpora cardiaca–CA complexes had set aside a bioactive fraction B, having higher apparent molecular mass (≈2.1 kDa) and ionic character (6). We indicated (6) that this was a major fraction (one-half to two-thirds) of the bioactivity found in crude brain extracts. Moreover, a study of the changing sensitivity of the CA towards ASA1 showed that glands from 5-day-old mated females, at their peak of JH-dependent vitellogenesis, gave no response to 10 nM ASA1 (8). This result left unexplained the significant allatostatic sensitivity of CA from vitellogenic females toward modest concentrations of unfractionated brain extracts (12).

Clearly, a quantitatively major allatostatin with a qualitatively different physiological function must be present in cockroach brains. Indeed, neither the limited potency of the smaller allatostatins (5) nor the developmental pattern of sensitivity of the glands to ASA1 (8) can account for the observed effects of crude brain extracts (12).

Here we report the identification of an octadecapeptide amide (ASB2) having strong C-terminal homology with type A allatostatins and a potential dibasic processing site at positions 9 and 10. We also report studies on partial sequences that indicate that the tyrosine-rich N terminus confers additional allatostatic potency, especially during vitellogenesis and early-middle pregnancy. We suggest that this peptide is a second type of allatostatin and that the putative cleavage site can escape tissue-specific proteolysis.

MATERIALS AND METHODS

Extraction and Prepurification of Allatostatin ASB2. Batches of brains and retrocerebral corpora cardiaca–CA complexes from 10- to 12-day-old mated females were extracted in acid ethanol and processed through C_{18} and diol Sep-Pak cartridges exactly as described for the isolation of type A allatostatins (6). The recovery of biological activity was assessed in allatostatic units, representing the quantity

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Abbreviations: JH, juvenile hormone; CA, corpora allata; ASB2, allatostatin B2; ASA1, allatostatin A1.

that causes 50% inhibition of JH biosynthesis in a standard radiochemical assay with CA from 10-day-old mated females (6).

HPLC. Reversed-phase HPLC was performed by using the same RP4 and RP18 columns (Pierce) and general procedures as used for the isolation of ASA1 (6, 7). The RP4 column was eluted with a gradient of 10–50% CH_3CN (1% per min at 1 ml/min) with isocratic modifiers (1% formic acid/0.3% triethylamine). The RP18 column was eluted (0.1% trifluoroacetic acid; 0.2 ml/min) by using a gradient of 10–50% CH_3CN (2% per min).

Resolution of Leu^{18}/Ile^{18} isomers of synthetic ASB2, and analysis of natural ASB2 peptide was achieved on a 4.6 × 25 mm Zorbax Rx(C_8) column (Rockland, Gilbersville, PA; 1.5 ml/min, 0.1% trifluoroacetic acid) fitted with a Rheodyne 7125 injector and Peek tubing, with a gradient of 20.25–24% CH_3CN (0.09375% per min). Fractions for bioassay were collected manually every 30 sec into siliconized tubes preloaded with bacitracin (30 μg) and crystallized bovine serum albumin (200 μg).

Physicochemical Analyses. Amino acid composition of hydrolyzates was performed with a Beckman 6300 autoanalyzer using post-column ninhydrin detection. N-terminal Edman sequencing was performed on an Applied Biosystems 470A gas–phase sequencer leading to an ABI 120A phenylthiohydantoin analyzer. Molecular masses were determined on a ZAB-SE MS (VG Analytical, Manchester, U.K.) in fast atom bombardment positive mode. Amino acid sequence analysis was done on a quadrupole Fourier transform MS and on a Finnigan TSQ 70 triple-quadrupole MS. Operation of both the above instruments for the analysis of peptides has been described (13–15).

Peptide Synthesis. Authentic peptides were synthesized on an Applied Biosystems model 430A synthesizer and purified by reversed-phase liquid chromatography. For each peptide, the content and composition of the lyophilized products were confirmed by amino acid compositional analysis and fast atom bombardment MS on a VG ZAB-SE instrument. N^{α}-acetylated peptides were prepared by treatment of the resin-bound peptide with pyridine/acetic anhydride, 1:1 (vol/vol) for 2 hr, essentially as described (16).

Analysis of Dose–Response Curves. The values of mean inhibition (%) and associated errors were obtained for each treatment from two to four replicates of groups of six individually incubated pairs of CA with an equal number of concurrent control incubations used as reference. These errors are proportional to the observed rate of synthesis (8); hence the precision of estimated inhibition decreases at low levels. Inhibition was normalized on the observed maximum in each case (range, 88–95%). IC_{50} values were derived from center-weighted least-squares regression analysis (Statgraphics) of the logistic (17). CA from 10-day-old mated females were used unless noted otherwise.

RESULTS

Isolation of Allatostatin ASB2. Initial purification of allatostatins from brain extracts by C_{18} Sep-Pak followed by diol Sep-Pak separated type A and type B allatostatins (6, 7). The lower-molecular-mass type A allatostatin was identified as a tridecapeptide ASA1 (6). The higher-molecular-mass type B allatostatins, which eluted from diol Sep-Pak only in 0.1% trifluoroacetic acid, were fractionated into ASB1 and ASB2 components by reversed-phase HPLC. The ASB1 bioactivity was variable from batch to batch, of lower hydrophobicity than either ASA1 or ASB2, and was not identified. The ASB2 fraction was more hydrophobic than type A allatostatins and was repeatedly the major type B activity, eluting from the RP4 column at 24 ± 0.5% CH_3CN. Further purification was achieved by RP18 HPLC with diode-array UV-effluent monitoring, which suggested that ASB2 was a tyrosine-rich peptide (>700 pmol of tyrosine per 6000 extracted brains) contaminated with nonaromatic impurities. These impurities were removed by rechromatography on a shallower gradient (30–40% CH_3CN; 0.25%/min). Fig. 1 shows one of three final purifications of ASB2 from which the two most bioactive fractions were taken for chemical analysis. The overall yields of ASB2 activity (≈1.2 allatostatin units per extracted brain) were one-third those of ASA1 (6). The ratio of specific bioactivity to UV absorption at 276 nm (representing primarily tyrosine, because the low-end absorption indicated tryptophan to be absent) was ≈five times that obtained from purified ASA1. In fact, subsequent analysis, synthesis, and reevaluation (see below) showed that ASB2 has an identical molar specific activity to ASA1 in standard (day 10) bioassays but had four times the molar tyrosine content.

Structure Determination. Three preparations of spectrally pure ASB2, from 6000, 3500, and 630 extracted brains, were used to elucidate the primary structure of ASB2. We obtained an average molecular mass of 2168.7 by fast atom bombardment MS and a partial (2–12) N-terminal sequence by gas-phase Edman degradation (yield in pmol indicated in parentheses): Xaa-Tyr (7.7) -Ser (6.3) -Tyr (3.9) -Val (3.7) -Ser (3.0) -Glu (3.7) -Tyr (3.1) -Lys (2.6) -Arg (4.3) -Leu (1.3) -Pro (0.6)-. The determined yields for selected, stable phenylthiohydantoin–amino acid derivatives versus sequencer cycle number were subjected to a linear regression analysis to determine the initial and average repetitive yield values for the analysis. These values were determined to be 12.0 pmol and 81.3%, respectively. This sequence analysis suggested a peptide of 17–19 residues with an N-terminal segment radically different from the known A allatostatins and explained the strong UV signal due to tyrosine observed during HPLC.

Another preparation, containing an estimated 122 pmol, was divided into three aliquots. One aliquot was employed to record a collision-activated dissociation mass spectrum on the $[M+H]^+$ ion centered at m/z 2169. A metastable decomposition spectrum of the same $[M+H]^+$ ion was recorded on aliquot two by using the quadrupole Fourier transform instrument. Fragmentation in both spectra was sufficient to define the N-terminal residue as alanine and to confirm the 12-residue sequence obtained by Edman degradation. To generate sequence information at the C terminus of the peptide, the third aliquot of the sample was digested with trypsin, and a fragment showing an $[M+H]^+$ ion at m/z 921 was isolated by HPLC. Attempted conversion of this fragment to the corresponding methyl ester failed to shift the observed $[M+H]^+$ ion (m/z 921) to higher mass. Absence of

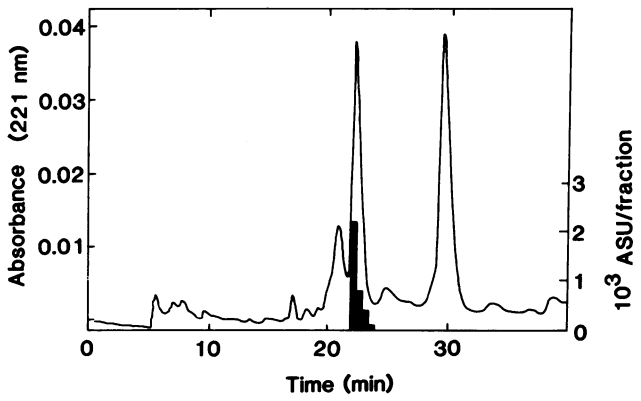

FIG. 1. Final-stage purification of allatostatin ASB2 from 3500 brain–corpora cardiaca–CA complexes by RP18 HPLC with a 30–40% CH_3CN gradient. Fractions (0.1 ml) were collected every 30 sec, and the bioactivity of 1% aliquots is expressed in allatostatic units (ASU) per fraction.

carboxylic acid groups in the sample dictated that the peptide contain a blocking group at the C terminus. The sequence Lxx-Pro-Val-Tyr-Asn-Phe-Gly-Lxx-NH_2 (where Lxx = Leu or Ile) was deduced from a collision-activated dissociation mass spectrum recorded on the $[M+H]^+$ ion at m/z 921. Leucine and isoleucine, two amino acids of identical mass, cannot be distinguished as yet on the triple-quadrupole instrument. Presence of one of these residues in the above tryptic peptide corresponds to residue 11 deduced by Edman degradation. Because the mass of residues 1–12 and the last six residues in the tryptic peptide sum to that of the intact peptide $[M+H]^+$ ion at m/z 2169, we concluded that the allatostatin ASB2 is an octadecapeptide amide. The Leu^{18} and Ile^{18} variants of ASB2 were synthesized, and both elicited a maximal response on CA from 10-day-old mated females with an IC_{50} within one order of magnitude of that seen with ASA1 (Table 1). The two synthetic peptides cochromatographed with natural product ASB2 on our final-stage HPLC (data not shown).

We resolved the leucine/isoleucine uncertainty by developing a higher resolution reversed-phase HPLC system that afforded base-line separation of the two isomers of ASB2. Fig. 2 shows that all significant bioactivity had a retention time identical to that of the slower-eluting Leu^{18} isomer, which completes our proof that allatostatin ASB2 has the primary sequence: Ala-Tyr-Ser-Tyr-Val-Ser-Glu-Tyr-Lys-Arg-Leu-Pro-Val-Tyr-Asn-Phe-Gly-Leu-NH_2.

We assume that the C-terminal amidations are derived from a glycine residue in the prohormones, through the action of peptide-amidating monooxygenase enzyme (11). We applied probability data for the occurrence of individual residues in omega loops of proteins (18) to all five allatostatin C termini and found that the six residues (C-terminal pentapeptide-glycine) represent a potential omega loop with criterion equal to 1.1 or greater. Thus, the allatostatins join a substantial group of invertebrate and vertebrate hormones that comply with the heuristic motif for omega loops at a site adjacent to endoproteolytic cleavage of their prohormones (19). The N-proximal (2–8) heptapeptide of ASB2 also represents a potential omega loop (criterion equal to 1.1), but the N-terminal composition of type A allatostatins does not predict the presence of an omega loop.

Gland Sensitivity During Reproductive Cycle. We performed dose–response curves with pure synthetic ASB2 at several ages during the first reproductive cycle for comparison with our previous results (8), which had shown major changes in glandular sensitivity toward allatostatin ASA1. Although ASA1 and ASB2 exhibit almost identical activities

Table 1. Bioactivity of allatostatins ASB2, ASA1, and related peptides

Peptide	pIC_{50}*
ASB2	9.51 (0.23)
ASA1	9.68 (0.21)
[Ile^{18}]-ASB2	8.79 (0.14)
[Asp^6,Ser^{10}]-ASA1-(6–13)-octapeptide amide	8.12 (0.15)
ASB2-(9–18)-decapeptide amide	6.48 (0.54)
ASB2-(10–18)-nonapeptide amide	6.88 (0.13)
ASB2-(11–18)-octapeptide amide	7.32 (0.11)
[D-Pro^{12}]-ASB2-(11–18)-octapeptide amide	7.34 (0.11)
[Arg^{12}]-ASB2-(11–18)-octapeptide amide	6.83 (0.18)
Ac-ASB2	9.19 (0.39)
Ac-ASB2-(9–18)-decapeptide amide	7.35 (0.19)
Ac-ASB2-(10–18)-nonapeptide amide	7.68 (0.02)
Ac-ASB2-(11–18)-octapeptide amide	7.74 (0.19)

*pIC_{50} is the negative logarithm of the molar concentration of peptide causing half-maximal inhibition of JH synthesis in the 2-hr bioassay with CA from 10-day-old mated females. Values in parentheses are SEs. Ac, N^{α}-acetylation.

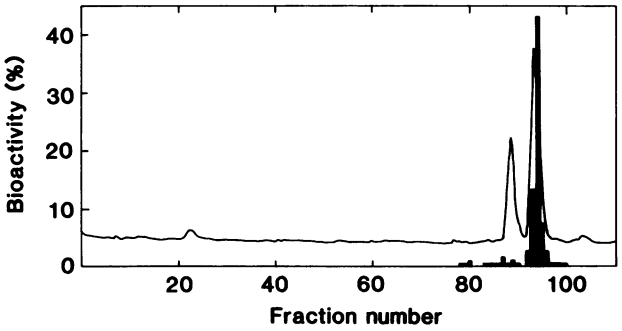

FIG. 2. Resolution of Leu^{18}/Ile^{18} isomers of synthetic ASB2 by RP8 HPLC with a 20.25–24% CH_3CN gradient. Natural product from 630 brain complexes was localized by bioassay of 9% aliquots of 0.6 ml fractions (black histogram); results are expressed as percentage recovery of the bioactivity applied to the column (total recovery, 78%). The UV profile (——) of the ASB2 isomer standards was obtained at 276 nm.

toward CA from 5-day-old virgin females (ASA1 pIC_{50}, 9.59 ± 0.43; ASB2 pIC_{50}, 9.52 ± 0.28) and 10-day-old pregnant females (Fig. 3), developmental profiles of the responses to 10 nM peptide reveal a more uniformly high response to ASB2 than to ASA1 in mated females (Fig. 4).

For instance, ASB2 has just over twice the activity of ASA1 at 10 nM against CA from 2-day-old mated females (Fig. 3). Nevertheless, ASB2 cannot completely suppress JH synthesis during the first phase of reproduction (65% in our standard 2-hr assay), and there is still a significant drop in sensitivity to ASB2 at the peak of vitellogenesis and spontaneous CA activity on days 4 and 5 (Fig. 4). This profile is closely comparable with one previously obtained by using whole brain extracts (12); thus, ASB2 should be considered a major active principle. Coadministration of allatostatins ASA1 and ASB2 at 10 nM to CA from 2-day-old mated females did not result in additive inhibition, confirming that ≈30% of the pathway flux in a standard bioassay is independent of allatostatin regulation at this time.

Site of Action of Allatostatins. We found that ASB2 acts at an early stage in the biosynthetic pathway, at least in the case of sensitive CA from 10-day-old mated females. Coincubation with the exogenous JH precursor (*E, E*)-farnesol (200 μM) reversed the inhibition caused by all concentrations of ASB2 over the range 1 nM–1 μM (84–92% reversal of inhibition), as we have shown with ASA1 (6). We also observed substantial reversal of inhibition with 5 mM (*R, S*) mevalonate coadministered with 10 nM ASB2 (64% rescue), 10 nM ASA1 (56% rescue), and 100 nM *N*-acetyl-ASA1 (72%

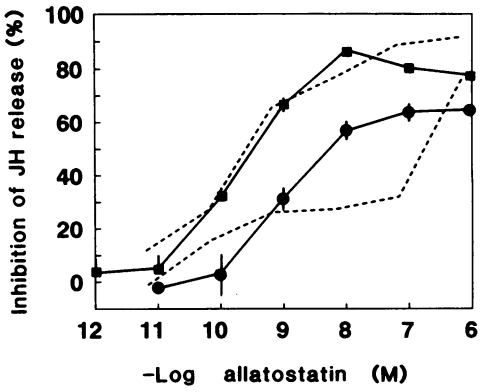

FIG. 3. Activities of synthetic ASB2 (——) and ASA1 (– – –, redrawn from ref. 8) on CA from 10-day-old (■) or 2-day-old (●) mated females. Inhibition of JH release *in vitro* was determined in 2-hr bioassays. Vertical bars indicate SEM (n = 12 or 18).

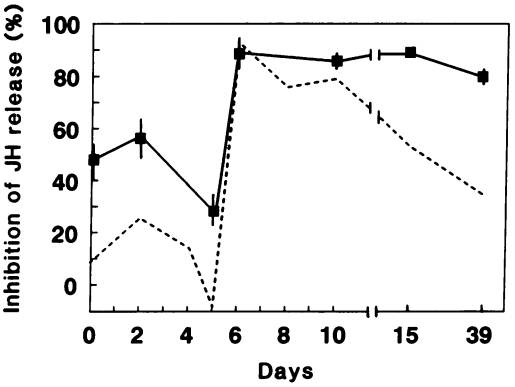

FIG. 4. Changes in sensitivity to 10 nM ASB2 throughout the first reproductive cycle. Data represent the mean inhibition of JH release in 2-hr bioassays of 12 individual pairs of CA from mated females of each age. – – –, Sensitivity to 10 nM ASA1 under the same experimental conditions (8).

rescue). In view of the known limited ability of exogenous mevalonate to enter the biosynthetic pathway (20), these results indicate that the pathway target(s) for both type A and type B allatostatins are before mevalonate kinase. However, this does not establish whether both allatostatin types target the same enzyme(s) or act via the same intracellular messengers.

Bioactivity of Partial Sequences. The C-terminal hexapeptide moiety of ASB2 has a high degree of sequence homology with type A allatostatins (Fig. 5). This C-terminal differs only by a conservative substitution of Val^{13} for a leucine in all type A allatostatins and a substitution of Asn^{15} for glycine, alanine, or serine. None of the shorter allatostatins can be generated by proteolysis of ASB2. It is known that the Phe^{11}, Leu^{13}, and terminal amide groups in ASA1 are independently essential for bioactivity (7). To test for functional homology of the C termini of the two types of allatostatins, we synthesized the N-terminal segments ASB2-(1–9)-nonapeptide amide and ASB2-(1–11)-undecapeptide amide and found them to be completely inactive. Moreover, the des-Leu^{18}-ASB2 had no bioactivity at 1 μM.

We also coadministered ASB2-(1–9)-nonapeptide amide (10 μM) with ASB2-(11–18)-octapeptide amide (10 and 100 nM), ASB2 (10 nM), and ASA1 (10 nM): in no case did the separate address moiety of ASB2 have any effect on the activity of the message-containing fragment or complete hormone. It seems that the nine-residue N terminus of ASB2 has no independent action on the CA.

We synthesized a "hybrid" octapeptide [Arg^{12}]-ASB2-(11–18)-octapeptide amide to introduce some A-type hydrophilicity at this site, and also the [D-Pro^{12}]-ASB2-(11–18)-octapeptide amide as a control for conformational perturbation. The two proline stereoisomers had identical activity, but the Leu^{11}-Arg^{12} combination was markedly disadvantageous (Table 1). This result indicated to us that the hydrophobic Leu^{11}-Pro^{12} pair of ASB2 has a type-specific addressing function that distinguishes it from the more hydrophilic type A allatostatins (corresponding, e.g., to Gln^{6}-Arg^{7} of ASA1 and Asp^{1}-Arg^{2} of the type A octapeptide amide).

N$^{\alpha}$-acetylation of ASB2 caused a small loss of activity, but it partially restored that of the truncated (9–18), (10–18), and (11–18) ASB2 amides (Table 1). Apparently, the greater the formal positive charge on these partial sequences, the greater the loss of bioactivity and the greater the extent of restoration by N$^{\alpha}$-acetylation.

DISCUSSION

We have identified a major active principle from cockroach brains capable of inhibiting the synthesis of JH by CA *in vitro* in a manner qualitatively and quantitatively different from the shorter and less potent allatostatins known to date (5–8). These findings necessarily increase the complexity of any model purporting to explain the physiological mechanisms of regulation of the CA (1, 21) and leave many new questions unanswered. The apparent existence of two types of allatostatic peptides, with at least four members of the A type, may suggest biological redundancy or may have its origins in the variety of environmental and physiological parameters that are integrated by the brain–ovary axis (1). Mating, feeding, and ovarian cyclicity are clearly distinct influences on CA activity (1). Thus, each type or species of allatostatin may be produced in different neurosecretory cells implementing specific physiological responses upon release from neurosecretory terminals within the CA (22). This can be tested, once type-specific antibodies are available.

Regarding the possible role of allatostatins in adult females, ASA1 appears to be mainly associated with the rapid inhibition of the CA at the end of vitellogenesis (8), probably as part of a response to unidentified signal(s) generated in the ovary (23). We find that ASB2 can inhibit JH synthesis of the CA from females in early vitellogenesis by 65%, but the inhibition is not complete even at saturation. Perhaps the type B allatostatins arose as messengers of nutritional insufficiency, which has a profound effect on CA activity in some situations (1, 21), and the limited sensitivity we have observed is a relic, relating to the fact that the first gonotrophic cycle in *Diploptera* is largely independent of adult nutrition (24). Comparative studies on a cockroach such as *Periplaneta americana* or *Leucophaea maderae*, whose CA are strongly influenced by starvation in the adult female (25, 26), may help answer this question.

Despite the quantitative differences in CA sensitivity toward type A versus type B allatostatins during the first reproductive cycle (Fig. 4) there remains a common pattern: the inverse relationship between the activity of the CA and their sensitivity to allatostatins. In particular, when the glands are at the height of synthetic activity during middle-late vitellogenesis (day 5), they cannot be inhibited by 10 nM

ASB2	Ala-Tyr-Ser-Tyr-Val-Ser-Glu-Tyr-Lys-Arg-Leu-Pro-Val	Tyr	Asn	Phe-Gly-Leu-NH_2
ASA1 (1)	Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu	Tyr	Gly	Phe-Gly-Leu-NH_2
(2)	Gly-Asp-Gly-Arg-Leu	Tyr	Ala	Phe-Gly-Leu-NH_2
(3)	Gly-Gly-Ser-Leu	Tyr	Ser	Phe-Gly-Leu-NH_2
(4)	Asp-Arg-Leu	Tyr	Ser	Phe-Gly-Leu-NH_2

FIG. 5. Alignment of allatostatin primary structures. ASB2, this work; ASA1, refs. 5 and 6; numbers in parentheses indicate the type A allatostatins reported in ref. 5. Residues common to all five peptides are boxed.

ASA1 (8), and 10 nM ASB2 causes only a 30% inhibition. Clearly, the physiological role of allatostatins as rapidly reversible regulators of JH biosynthesis is itself subordinate to the currently unknown factors that modulate CA sensitivity as well as JH synthetic rates.

Probable Integrity of the Potential Dibasic Cleavage Site. The presence of the N-terminal 1–10 segment of ASB2 markedly enhances the allatostatic potency, especially toward CA from vitellogenic females. However, these studies do not eliminate the possibility that some cells process ASB2 as a prohormone at the Lys^{9}-Arg^{10} dibasic site. In that event, the bioactivity data (Table 1) coupled with the typical influence of proline residues on amino-monopeptidases (27), suggest that the hormonally active product would be the ASB2-(11–18)-octapeptide amide. We addressed this possibility by examining the effects of N^{α}-acetylations on bioactivity. We reasoned that if a peptide interacts with a receptor that is specific for it, then N^{α}-acetylation of the peptide may reduce its efficacy because of steric and/or charge effects. Conversely, if the peptide is unnaturally shortened, then N^{α}-acetylation should (*i*) cause no steric repulsion and (*ii*) eliminate the unnaturally located positive charge on the backbone.

Our results (Table 1) are consistent with ASB2 being a natural hormone, but it would be premature to conclude that the ASB2-(11–18)-octapeptide amide merely binds with much reduced affinity to the octadecapeptide receptors. We cannot exclude the possibility that it interacts with a different receptor type having a much smaller binding site. It is noteworthy (Table 1) that the ASB2-(11–18)-octapeptide amide is six times less active in these assays than the type A octapeptide that has already been identified as a natural product (5, 7).

We expect the dibasic site in ASB2 to be prone to cleavage by a variety of tissue endoproteases. As found here (Table 1), this may result in a 100- to 200-fold loss of bioactivity, which could explain the apparent absence of this peptide from saline aqueous extracts of whole brains (5).

The existence of two types of allatostatins sharing a conserved message sequence increases the burden of proof required to elucidate their modes of action at anatomical-through-molecular levels during larval development and adult reproduction. Selective probes will be needed to isolate and quantify allatostatin receptors, whose levels may be important determinants of CA sensitivity. Isolation of the allatostatin gene(s) that hold the secrets of the molecular history of these important regulatory peptides awaits further research.

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