IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A PUTATIVE ALLATOSTATIN RECEPTOR IN THE MIDGUT OF THE COCKROACH *DIPLOPTERA PUNCTATA*

by

Paul R.F. Bowser

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Zoology
University of Toronto

© Copyright by Paul R.F. Bowser 1999
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-45564-5
Identification and partial characterization of a putative allatostatin receptor in the midgut of the cockroach Diploptera punctata

Paul R. F. Bowser

M.Sc. 1999

Department of Zoology, University of Toronto

Abstract

A putative receptor for the peptide Dip-allatostatin 7 (Dip-AST 7) was identified in the midgut of the cockroach, Diploptera punctata and was partially characterized using a radioligand-binding assay. The midgut contains a single binding site for Dip-AST 7 with $K_d$ of $14\pm2.2$ nM and $B_{\text{max}}$ of $1.5\pm0.1$ pmol/mg membrane protein. Binding was saturable, specific and reversible and is physiologically consistent with a receptor. The relative affinity of other members of the Dip-allatostatin family of peptides was determined using a single-point competitive binding assay. The rank order of competitive potency of the allatostatins tested was: Dip-AST 7 > Dip-AST 2 > Dip-AST 5, 9, 11 > Dip-AST 13. A synthetic pseudopeptide analog of allatostatin, AST($b$)$\Phi$2, was not an effective competitor for the putative allatostatin receptor.
Acknowledgements

I would like to thank Steve Tobe for his constant support, and for letting me try things my own way; my parents, for their patience and for helping me cover tuition; everyone in the lab for making it fun to come to work every day; and the Orchard lab (and Leandra) for always knowing the perfect time to suggest a drink.
Table of contents

ABSTRACT ........................................................................................................... II

ACKNOWLEDGEMENTS .......................................................................................... III

TABLE OF CONTENTS ............................................................................................ IV

LIST OF FIGURES, EQUATIONS AND TABLES ....................................................... VI

LIST OF ABBREVIATIONS ....................................................................................... VII

1. INTRODUCTION ................................................................................................. 1

ACTIVITIES OF THE DIP-ALLATOSTATINS .......................................................... 1

  Inhibition of Juvenile Hormone biosynthesis ...................................................... 2
  Inhibition of visceral muscle contraction ............................................................ 3
  Effects on digestive enzyme secretion ............................................................... 4
  Allatostatin as a neurotransmitter / neuromodulator ......................................... 6

ALLATOSTATIN RECEPTORS .................................................................................. 7

  Signal transduction ............................................................................................ 7
  Multiple allatostatin receptors ........................................................................... 9

STRUCTURE-ACTIVITY RELATIONSHIPS ............................................................ 10

METABOLISM OF THE ALLATOSTATINS ............................................................ 11

OCURRENCE AND EXPRESSION OF ALLATOSTATINS IN THE MIDGUT ............. 13

  Structure of the cockroach midgut .................................................................... 13
  AST-immunoreactive 'endocrine' cells ............................................................... 14
  Nerve fibres ...................................................................................................... 14

PURPOSE OF THIS STUDY ....................................................................................... 16

2. MATERIALS AND METHODS ........................................................................... 17

ANIMALS ................................................................................................................ 17

CHEMICALS .......................................................................................................... 17

RADIOACTIVITY MEASUREMENT ...................................................................... 17

PREPARATION OF 125I-DIP-AST ...................................................................... 18

PURIFICATION OF 125I-DIP-AST ........................................................................ 18

MASS SPECTROMETRY ......................................................................................... 19

DETERMINATION OF SPECIFIC RADIOACTIVITY ........................................... 19

PREPARATION OF MIDGUT PLASMA MEMBRANES ........................................ 20
List of Figures, equations and tables

FIGURES
1. HPLC separation of Dip-AST 7 iodination products ......................................................... 23
2. Ion-electrospray mass spectrometry .................................................................................. 26
3. Quantitation of specific radioactivity by self-displacement RIA ........................................ 28
4. Saturable binding to midgut plasma membrane by Dip-AST 7 ........................................... 31
5. Reversibility of Dip-AST 7 binding ..................................................................................... 33
6. Competitive potency of selected Dip-ASTs ....................................................................... 36
7. Competitive potency of a pseudopeptide Dip-AST analog .................................................. 38
8. Comparison of standard and single-point competitive binding assays ................................ 47

TABLE
1. Relative competitive potency and amino acid sequence of selected ASTs ....................... 51

EQUATIONS
1. Binding to a single class of sites ......................................................................................... 21
2. Binding to two classes of sites ......................................................................................... 21
3. Receptor occupancy ........................................................................................................ 43
List of Abbreviations

Ab............antibody
ACN............acetonitrile
Ala............alanine
Arg............arginine
Asn............asparagine
Asp............aspartate
AST............allatostatin
ASTR............allatostatin receptor
ATP............adenosine triphosphate
B_max............maximum binding
Bq............Bequerel
BSA............bovine serum albumin
Ca^{2+}............calcium ion
CA............corpora allata
cAMP............cyclic adenosine monophosphate
cGMP............cyclic guanosine monophosphate
Ci............Curie
cpm............counts per minute
Cys............cysteine
DAG............diacyl glycerol
Da............Dalton
Dip-AST...........Diploptera punctata allatostatin
DNA............deoxyribonucleic acid
ELISA............Enzyme-linked Immunosorbent Assay
fmol............femtomoles (10^-15 moles)
G-protein............guanosine triphosphate binding protein
Gly............glycine
GTP............guanosine triphosphate
h.............hour
HPLC............high performance liquid chromatography
I............iodine
Ile............isoleucine
IP_3............inositol triphosphate
JH............juvenile hormone
K^+............potassium ion
K_D............dissociation constant
kDa............kilodalton
Leu............leucine
Lys............lysine
Met............methionine
min............minute
mM............millimolar (10^-3 M)
M............molar
M_r............relative molecular mass
Mg^{2+}............magnesium ion
Mn^{2+}............manganese ion
mRNA............messenger ribonucleic acid
nM............nanomolar (10^-9 M)
NaOH............sodium hydroxide
PKC............protein kinase C
pmol............picomoles (10^-12 moles)
PMSF............phenylmethylsulfonyl fluoride
RIA............radioimmunoassay
RNA............ribonucleic acid
RT............room temperature
SDS-PAGE...........sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser............serine
t_1/2............half-life
TFA............trifluoroacetic acid
Trp............tryptophan
Tyr............tyrosine
µl............microlitre (10^-6 litres)
µM............micromolar (10^-6 M)
Xaa............variable amino acid
1. Introduction

Allatostatins (ASTs) are a family of peptides first isolated from the cockroach *Diploptera punctata* (Woodhead *et al.* 1989) and now known to be widely distributed among insect orders (see Bendena *et al.* 1997). In cockroaches, ASTs have a number of different functions. Their most well documented role is the inhibition of juvenile hormone (JH) production by endocrine glands called the corpora allata (CA) (see Tobe *et al.* 1994). ASTs also inhibit spontaneous and proctolin-induced contractions of hindgut and foregut visceral muscle (Lange *et al.* 1993, 1995; Duve *et al.* 1995). Recently, they have been implicated as modulators of digestive enzyme production by the midgut (Fusé *et al.* 1998). Putative receptors for ASTs in several tissues of cockroaches, including CA, brain, and hindgut, have previously been characterized using a radioligand-binding assay (Yu *et al.* 1995a; Yu, Bendena and Tobe, unpublished data).

*Activities of the Dip-allatostatins*

ASTs appear to have a variety of different biological roles. They were originally isolated based on their ability to inhibit the biosynthesis of JH by the CA of cockroaches (Tobe and Stay 1985). More recently, it has been demonstrated that ASTs also have other activities, including effects on muscle contraction (Lange *et al.* 1993, 1995; Duve *et al.* 1995) and enzyme secretion (Fusé *et al.* 1998), and likely have a role as neuromodulators (Stay *et al.* 1994). The AST family in *D. punctata* includes 13 peptides, encoded by a single gene (Donly *et al.* 1993). Modern nomenclature for ASTs is based on the species of origin and the sequence of peptides on the gene (Raina and Gade 1988; Donly *et al.* 1993). For example, Dip-AST 7 is the seventh peptide coded for by the *Diploptera punctata* AST gene. The seven ASTs that were isolated prior to the sequencing of the AST gene are still occasionally referred to by a “classic” nomenclature based
on the order in which they were isolated. For example, the AST currently referred to as Dip-AST 7 was the first AST isolated, and is thus sometimes called AST I (Woodhead et al. 1989).

**Inhibition of Juvenile Hormone biosynthesis**

In most insect species, JH is an important regulator of metamorphosis and reproduction (Wigglesworth 1964; Steel and Davey 1985). JH is normally required for the maintenance of juvenile characteristics and its absence may be necessary for the final molt from larva to adult (Granger et al. 1981). In adult insects, JH serves as a regulator of vitellogenesis and thus plays a vital role in reproduction (Steel and Davey 1985). In *D. punctata*, oocyte growth can be directly correlated with increasing JH titer during the first part of the gonotrophic cycle (Tobe and Stay 1985). Successful development and reproduction in most insect species therefore requires precise control of JH titer. In mated female *D. punctata*, JH biosynthesis follows a distinct cycle. Synthesis is low for the first few days after adult emergence, but increases dramatically on day 4, reaching a peak on day 5 and declining rapidly thereafter (Tobe and Stay 1977; Tobe 1980). Sensitivity of the CA to ASTs varies during this cycle. When JH biosynthetic activity is at its peak, low concentrations of ASTs do not inhibit JH biosynthesis. One day later, maximal inhibition (85-90%) can be elicited with the same concentration of AST. Changes in sensitivity may be the result of modulation of receptor density, affinity, or of changes in receptor subtype ratios (Stay et al. 1994). The fundamental mechanism for AST inhibition of JH biosynthesis is not fully understood, but it may involve the transport of 2-carbon units as citrate across the mitochondrial membrane to the cytosol by ATP-citrate lyase (Sutherland and Feyereisen 1996). This transport and the subsequent cleavage of citrate provide the substrate (acetyl-CoA) that is generally considered to be the first committed step of the JH III biosynthetic pathway (Schooley and Baker 1985). Other evidence suggests that ASTs inhibit JH biosynthesis at least partially by
inhibiting the activity of enzymes involved in terminal stages of the biosynthetic pathway (Wang et al. 1994). Dip-AST 5 and a related peptide from Calliphora vomitoria, callatostatin 5, both appear to modify the activity of o-methyltransferase and epoxidase, the enzymes that catalyze the final steps of JH biosynthesis. It is possible that ASTs inhibit the JH biosynthetic pathway at multiple levels, which could permit a redundancy of control.

Inhibition of visceral muscle contraction

ASTs also inhibit both myogenic and proctolin-induced contractions of the hindgut (Lange et al. 1993, 1995) and foregut (Duve et al. 1995) of cockroaches. Interestingly, when ASTs are ranked in order of potency, the order as inhibitors of hindgut contraction is approximately opposite to that for inhibition of JH biosynthesis (Lange et al. 1995; see Bendena et al. 1997 for rank order of ASTs as inhibitors of JH biosynthesis). Dip-AST 2, which is the most potent Dip-AST inhibitor of in vitro JH biosynthesis, ranks tenth (of thirteen) as an inhibitor of hindgut contraction. Conversely, Dip-AST 9, which is only a moderately potent inhibitor of in vitro JH biosynthesis (ranking eighth of thirteen), is the most potent inhibitor of hindgut contraction (Lange et al. 1995). Similarly, Dip-AST 13, which has the lowest potency for inhibition of JH biosynthesis, ranks third as an inhibitor of hindgut contraction. Generally, ASTs are less potent as inhibitors of hindgut contraction than as inhibitors of JH biosynthesis, requiring 10 to 100-fold higher concentrations to achieve half-maximal effects. The inhibition of hindgut contraction by ASTs provided the first clue that ASTs may be involved in the control of digestion. Yu et al. (1995b) suggest that ASTs might slow the passage of food through the gut, permitting improved absorption of nutrients or slowing digestion and metabolism during starvation. The hindgut of cockroaches is heavily innervated with AST-immunoreactive neurons (Yu et al. 1995b) and ASTs may therefore be delivered by a neural, rather than hormonal route. Indeed, whereas the
hemolymph concentration of ASTs can reach 2.4 nM in *D. punctata* (Yu *et al.* 1993), this is well below their minimum *ED*<sub>50</sub> (100 nM) in the hindgut assay (Lange *et al.* 1995).

**Effects on digestive enzyme secretion**

Recently, Fusé *et al.* (1998) have demonstrated that ASTs are able to affect the production of digestive enzymes by the midgut. Isolated midguts are incubated for fixed periods in the presence or absence of ASTs. The contents of the lumen and the homogenized tissue are then assayed for invertase and α-amylase activity. ASTs appear to increase the activity of both invertase and amylase in a dose-dependent fashion in the lumenal contents, but not in the tissue. So far, the results suggest that ASTs enhance the secretion of invertase and α-amylase from the midgut epithelium. However, the possibility that ASTs effect a more direct modification of enzyme structure (e.g. by phosphorylation) leading to enhanced intrinsic activity has not been ruled out. A mechanism for endocrine effects on insect gut, based on studies of mammals, has been proposed by Fujita *et al.* (1981). In this mechanism, “open”-type endocrine cells function as primary sensors that register the nutrient content of the gut. For example, it is suggested that soluble enzymes in the gut lumen undergo an allosteric change when their active site conjugates with their substrate. Receptors on the surface of “open”-type endocrine cells would possess specific receptors for the allosterically changed enzyme molecules. Downstream effectors of these receptors could then effect an appropriate response (Fujita and Kobayashi 1977). Endocrine cells of the “closed” type are believed to monitor the tension in the gut wall (Fujita and Kobayashi 1977). When the appropriate sensory input is detected, the cells release their contents. Electron-microscopic evidence indicates that secretory granules in cockroach midgut endocrine cells gather at the basal pole and are released basally, or occasionally at the sides of the cell, by exocytosis (Endo and Nishiitsutsuji-Uwo 1982). Experiments with ligated tissues support
the view that ASTs are released to the haemolymph side of the midgut (Yu et al. 1995b). Nearly 50% of the total midgut AST content is released during a six hour incubation. Approximately half of the AST is released spontaneously into the medium within the first 30 minutes of incubation. After 60 minutes, further spontaneous release is negligible, but subsequent release can be potentiated by treatment with high concentrations of potassium (56 mM). Whether this represents ASTs released from endocrine cells, or from nerves, was not determined (Yu et al. 1995b). Some researchers have reported small numbers of secretory granules clustering near the apical pole of vertebrate gut endocrine cells and have proposed that secretion of at least some compounds may be bipolar (Nilsson et al. 1987). ASTs secreted into the lumen by endocrine cells could probably make their way into the haemolymph; certainly, the reverse appears to be true — ASTs added to the incubation medium surrounding a ligated midgut are able to cause changes in enzyme activity within the lumen (Fusé et al. 1998). However, the insect midgut contains a wide variety of proteolytic enzymes that would rapidly degrade any apically-secreted ASTs, which would drastically limit their effect. In addition, ultrastructural studies of exocytosis in endocrine cells of the midgut of the cockroach Periplaneta americana detected characteristic exocytotic omega figures at the basal and lateral surfaces of the cells, but not at their apical faces (Endo and Nishiitsutsuji-Uwo 1982). The effects of secretions from midgut endocrine cells are probably paracrine, influencing neighboring digestive cells and possibly extending to nerve termini beneath the epithelium. Some of the released material might also enter the haemolymph. Most ASTs released into the haemolymph would be rapidly degraded by peptidases (Garside et al. 1997a). However, certain ASTs, such as Dip-AST 5, are relatively resistant to degradation by enzymes in the haemolymph. These ASTs might play a special humoral role, such as providing a low but constant level of inhibitory signal to the CA, rendering the glands more responsive to
stronger inhibitory signals delivered by the nerves (Tobe et al. 1995); or regulating the myotropic activity of the alimentary tract (Yu et al. 1995b). The AST content of the midgut is greatly elevated after a single day of starvation, and remains significantly elevated after three days without food or water (Yu et al. 1995b). This may represent a build-up of ASTs in the absence of the appropriate release signal. An alternate possibility is that increased production of ASTs by midguts of starved animals may serve to reduce muscle contractions, resulting in decreased metabolic activity (Yu et al. 1995b).

Allatostatin as a neurotransmitter / neuromodulator

D. punctata brains contain numerous AST-immunoreactive cells, many of which appear to terminate within the brain (Stay et al. 1994). In addition, putative receptors for ASTs have been partially characterized in the brain by SDS-PAGE and radioligand-binding assay (Cusson et al. 1991; Yu et al. 1995a). To date, this remains the sole evidence pointing to a role for ASTs as neurotransmitters and/or neuromodulators within the central nervous system of insects. In crustaceans, however, there is strong evidence suggesting that ASTs can act within the nervous system. Dip-ASTs applied to the stomatogastric ganglion of the crab, Cancer borealis, decrease the rhythm of both the gastric mill (Skiebe-Corrette et al. 1993) and pyloric regions (Skiebe and Schneider 1994) of the foregut wall. The rhythm of these muscles is controlled by the discharge of motor neurones of the stomatogastric ganglion (Marder and Hooper 1985). These results indicate that ASTs are able to act centrally to modify neuronal activity (Stay et al. 1994). In addition, Jorge-Rivera and Marder (1997) demonstrated that Dip-ASTs decrease stomatogastric neuromuscular transmission in C. borealis. Dip-AST 3 reduces the amplitude of nerve-evoked contractions, excitatory junctional potentials and excitatory junctional currents at both
cholinergic and glutamatergic neuromuscular junctions in the stomach musculature by reducing the postsynaptic actions of neurally released acetylcholine and glutamate.

*Allatostatin receptors*

ASTs are believed to act by binding to receptors in the plasma membrane. Experimental evidence for the existence of these receptors has been provided using SDS-PAGE and a radioligand-binding assay (Cusson *et al.* 1991; Yu *et al.* 1995a). AST receptors (ASTRs) are assumed to be members of the G-protein linked, seven transmembrane domain receptor superfamily, which includes most neuropeptide receptors described to date (Tobe *et al.* 1994). However, efforts to clone the receptor gene based on homology to other G-protein linked receptors have to date been unsuccessful (W.G. Bendena, pers. comm.).

*Signal transduction*

Signal transduction downstream of the receptor is believed to follow the phosphoinositide second messenger pathway (Rachinsky *et al.* 1994). Experiments with inhibitors and activators of PKC and with inhibitors of DAG kinase indicate that both DAG and PKC are likely signal transducers for ASTs. Although inhibition of JH biosynthesis has been correlated with increased levels of cyclic nucleotides within the cells of the CA (Tobe 1990), ASTs themselves do not appear to directly affect the production of cGMP or cAMP (Cusson *et al.* 1992). However, the ability of PKC inhibitors to block AST-mediated inhibition of JH biosynthesis suggests that cyclic nucleotide-dependent protein kinases may play a role in this process (Rachinsky *et al.* 1994). Full activity of ASTs may therefore depend on cross-talk between different receptor-coupled signal transduction pathways (Tobe *et al.* 1994). Cross-talk can produce an amplification of responses produced by separate, coincident signals within a cell (Selbie and Hill 1998).
JH biosynthesis is almost totally inhibited in the absence of extracellular calcium, and is strongly affected by pharmacological agents which modify the intracellular calcium concentration of the CA (Kikukawa et al. 1987). There is ample evidence that CA cell membranes contain voltage-sensitive calcium channels (Thompson and Tobe 1986; Thompson et al. 1990; McQuiston et al. 1990). Inorganic calcium channel blockers, such as Mn^{2+} and Mg^{2+}, can reverse the inhibitory effect of either brain extracts or high K\(^+\) concentration on JH biosynthesis (Rankin et al. 1986; Feyereisen and Farnsworth 1987). Similarities in the inhibitory effects of high K\(^+\) concentration and brain extracts led Feyereisen and Farnsworth (1987) to conclude that the inhibitory factor in brain extracts might be identical to that released in the CA by high K\(^+\) treatment. High K\(^+\) concentrations are now known to release Dip-ASTs from CA (Yu et al. 1995b). Thapsigargin, which mimics the effects of IP\(_3\) by releasing intracellular calcium stores, increases the biosynthesis of JH by the CA of day 4 mated females but not 2-day-old virgin females (Rachinsky et al. 1994). The effects of thapsigargin on JH biosynthesis are antagonized by AST. Analogues of cyclic nucleotides and an activator of PKC affect the electrical properties of the cell membrane in a manner which could increase the duration of calcium channel opening (McQuiston and Tobe 1991). PKC, cAMP, and cGMP may therefore modulate the influx of calcium into the cell, making the CA cell membranes a site of integration and crosstalk of second messenger systems. Many of the second messenger mechanisms related to JH biosynthesis appear to be stage-specific (Rachinsky and Tobe 1996). It is possible that part of the explanation for the rapid and profound changes in sensitivity to ASTs is a switch from the use of one second messenger pathway to another. It is also possible that multiple AST receptors may be linked to different second messengers, and these may be expressed differentially throughout development.
Multiple allatostatin receptors

The large number and tissue-specific differences in potency of ASTs has led to the suggestion that there may be multiple, tissue-specific subtypes of AST receptors (Yu et al. 1995a; Lange et al. 1995). Furthermore, since sensitivity of at least some target tissues to ASTs varies during the gonotrophic cycle, it has been predicted that there may be a concomitant modulation of receptor subtype populations. There is some evidence to support these hypotheses. Radioligand-binding experiments indicate that different tissues have binding sites for ASTs that differ in their affinity for a given AST. Yu et al. (1995a) have reported that Dip-AST 5 binds to a single binding site in brain with a $K_D$ of $9.0 \times 10^{-10}$ M and a $B_{\text{max}}$ of 2.2 pmol/mg membrane protein. Dip-AST 7 appears to bind to two distinct sites, with $K_D$ of $1.5 \times 10^{-9}$ M and $3.8 \times 10^{-9}$ M and $B_{\text{max}}$ of 7.2 and 11.4 pmol/mg protein, respectively. Only one binding site for Dip-AST 7 is apparent in the CA, with $K_D = 7.2 \times 10^{-10}$ M and $B_{\text{max}} = 1.7$ pmol/mg protein. Putative AST receptors have been demonstrated by SDS-PAGE of isolated membranes of brain and CA, using radiolabeled photoaffinity analogs of ASTs to identify bands representing putative receptors. Cusson et al. (1991) demonstrated the presence of 59- and 39-kDa proteins in CA that specifically bind the Dip-AST 7 photoaffinity analog $[^{125}\text{I}]$-ASA-AST-1. In the brain, a 41-kDa protein specifically bound the analog. Similarly, Yu et al. (1995a) showed that a 37-kDa protein was specifically labeled by the Dip-AST 5 photoaffinity analog $[^{125}\text{I}]$-RYBPA. The 41-kDa protein identified by Cusson et al. (1991) and the 37-kDa protein identified by Yu et al. (1995a) are likely the same protein, with slight differences in apparent relative molecular mass ($M_r$) due to the different analogs and buffer system used. Given the similarities in $M_r$, it seems possible that the 41-kDa putative receptor identified by Cusson et al. (1991) in the brain is the same as the 39-kDa protein
found in the CA. In this case, small differences in apparent molecular mass might be due to phosphorylation, glycosylation, or other modification of the base protein.

Pratt et al. (1997) have argued that analysis of the activities of a variety of synthetic AST variants also indicates that two receptor types are present in the CA. The dose-response curve for inhibition of JH release from day 10 CA in vitro by several Dip-AST 2 analogs is generally consistent with the interaction of a ligand with a single binding site. In day 2 CA, the analog dose-response curves can be interpreted as biphasic, which would suggest the additive responses of two separate binding sites. However, the response to authentic Dip-AST 2 is characteristic of only a single binding site on both day 2 and day 10, and the fit of the analog data to a biphasic model is far from ideal. Given the inherently high variability of CA response to ASTs, this type of analysis is of questionable value.

Structure-activity relationships

The D. punctata AST gene has been sequenced, and the gene structure suggests that it codes for thirteen different peptides (Donly et al. 1993). Seven of the predicted peptides have previously been isolated from D. punctata using classical purification methods (Woodhead et al. 1989; Pratt et al. 1991a,b; Woodhead et al. 1993). Each AST is characterized by a common Tyr/Phe-Xaa-Phe-Gly-Leu/Ile-NH₂ C-terminus (Xaa=Gly, Ala, Ser, Asp, Asn) and has a unique N-terminal sequence of variable length (Donly et al. 1993). The thirteen Dip-ASTs are separated into four groups (1-4, 5-10, 11, and 12-13) along the AST precursor by acidic spacer domains which serve to balance the charge of the molecule and may serve to separate the ASTs into functional groups. The characteristic pentapeptide C-terminus of ASTs is highly conserved and is believed to represent the core "message" sequence of these peptides (Hayes et al. 1994). By
itself, the pentapeptide C-terminus of Dip-AST 5 (YSFGL-NH₂) is able to inhibit JH biosynthesis in vitro, albeit with approximately 200-fold lower potency than the full peptide (Stay et al. 1991b). Despite its reduced potency, the pentapeptide retains full efficacy; it is able to induce a maximal response if it is applied at sufficiently high concentrations. Further truncation, deamidation, or extension of the C-terminus abolishes activity entirely (Pratt et al. 1991b). Substitution of the C-terminal Leu with Ala drastically reduces potency, whereas conservative substitution with Ile, as is found naturally in Dip-AST 13, produces a more modest reduction in potency (Hayes et al. 1994). Evidence from radioligand-binding assays supports the view of the pentapeptide C-terminus as the core sequence of ASTs. In brain membrane preparations, the pentapeptide binds specifically to putative AST receptors, albeit with greatly reduced affinity, whereas truncation of the C-terminal Leu-NH₂ from Dip-AST 5 completely abolishes binding (Yu, Bendena and Tobe, unpublished data).

Metabolism of the allatostatins

The influence of ASTs is limited in time and space by their half-life (t½). ASTs are degraded by peptidases in the haemolymph and on the surfaces of potential target tissues. These peptidases recognize specific structural motifs, and different members of the AST family are degraded at different rates. In the haemolymph, 5 μM Dip-AST 7 (APSGAQRLYGFLa) is rapidly cleaved by a putative endopeptidase, yielding the C-terminal hexapeptide LYGFLa (Garside et al. 1997a). Further cleavage by an apparent aminopeptidase truncates this metabolic product to the C-terminal pentapeptide. Dip-AST 9 (GDGRLYAFGLa) appears to be degraded by the same pathway, but Dip-AST 5 (DRLYSFGLa), which shares a similar C-terminal sequence, has a t½ almost 20 times longer. The relatively rapid degradation of Dip-AST 7 and 9 probably limits
their ability to exert other than a paracrine effect. Dip-AST 5 might plausibly have a more extensive humoral role, traveling through the haemolymph to distantly located target tissues (Garside et al. 1997a). In addition to degradation by haemolymph-borne enzymes, ASTs are also cleaved by peptidases associated with target tissues (Garside et al. 1997b). Physiological (nanomolar) concentrations of Dip-AST 5 are degraded at different rates by membrane preparations of various tissues. The pattern of degradation by tissues differs from that in the haemolymph. Initial cleavage of Dip-AST 5 at Gly-Leu yields the N-terminal heptapeptide DRLYSFG. This product is inactive as an inhibitor of JH biosynthesis. The half-life for this process varies from 22.8 minutes with brain membranes, to 84.5 minutes with midgut membranes. Recently, information about the metabolism of ASTs has been exploited to create peptidomimetic analogs, in which sites susceptible to degradation are replaced by non-peptide moieties (Nachman et al. 1997; Garside et al. 1997c). It is hoped that such "pseudopeptides" might serve as novel insecticides by affecting various systems while resisting the normal degradative enzymes. For example, the pseudopeptide AST(b)ϕ2 is a mimic of Dip-AST 6 in which the Gly7 of the membrane-peptidase sensitive Gly-Leu motif has been replaced with a cyclopropyl ring system, and the Tyr4 residue at the N-terminus has been replaced by hydrocinnamic acid to remove cleavage sites sensitive to haemolymph enzymes (Nachman et al. 1997; Garside et al. 1997c). AST(b)ϕ2 has a greatly increased half-life in haemolymph or crude membranes preparations, and retains potency as an inhibitor of JH biosynthesis (ED50=1.6 nM) despite the structural modifications (Garside et al. 1997c).
Occurrence and expression of allatostatins in the midgut

Structure of the cockroach midgut

The midgut of the cockroach *P. americana* is composed of an epithelial monolayer resting on a basal lamina, surrounded by a layer of radial muscles which are in turn enclosed in a layer of longitudinal muscle (Nishiitsuji-Uwo and Endo 1981). The structure of *D. punctata* midgut is similar, although some structural differences in the alimentary tracts of these distantly related cockroach species do exist (*e.g.* *P. americana* has eight gastric caecae (Nishiitsuji-Uwo and Endo 1981), whereas *D. punctata* has only two). Unlike the foregut and hindgut, the midgut is of endodermal origin and lacks a chitinous lining (Nishiitsuji-Uwo and Endo 1981). Rather, specific cells of the midgut secrete a chitinous matrix called the peritrophic membrane that encloses the midgut contents. The epithelium of the cockroach midgut is composed of three cell types: columnar, endocrine, and regenerative (stem) cells (Nishiitsuji-Uwo and Endo 1981). Regenerative cells are located in structures called nidi, which are located in the basal part of the epithelium. Columnar cells, which make up the majority of the cells in the midgut epithelium, and endocrine cells, which make up a small percentage of the cell population, are both derived from the stem cells of the nidus. Columnar cells appear to have a 21-28 day life span, gradually shifting laterally from the nidus as they age, replaced by new cells in a “pipeline” system. In contrast, endocrine cells appear to have a variable lifespan and may be renewed via a “random loss” system (Endo et al. 1983). Endocrine cells are located on the basal lamina and are intercalated individually among the columnar cells (Nishiitsuji-Uwo and Endo 1981). Most endocrine cells in the *Periplaneta* midgut are of the “closed” type, but some are of the “open” type, reaching the gut lumen with apical extensions. They may be pyramidal, bowl-, spindle-, or
bottle-shaped, and can be classified into at least four types by reference to the shape, size, and density of the endocrine granules they contain (Nishiitsutsuji-Uwo and Endo 1981).

**AST-immunoreactive ‘endocrine’ cells**

Dip-AST immunoreactive cells in the midgut of *Diplaptera* appear to be of the “open” type and are pyramidal in shape (Reichwald *et al.* 1994; Yu *et al.* 1995b). These cells are sites of AST mRNA expression and are unevenly distributed in different regions of the midgut. They are most dense toward the anterior of the midgut, forming a narrow band in the region where nerve fibres appear to terminate. A broad region of intermediate density is found toward the posterior of the midgut, whereas the mid-region of the midgut is only sparsely populated with these cells. The most anterior portion of the midgut, approximately one-quarter of the total length, is completely free of AST-immunoreactive cells (Yu *et al.* 1995b). In contrast, AST-immunoreactive endocrine cells in the midgut of the cockroach *Blattela germanica* are distributed evenly over the whole midgut epithelium (Maestro *et al.* 1998). The identification of these AST-immunoreactive cells as ‘endocrine’ is based primarily on their ultrastructure and immunocytochemistry (Reichwald *et al.* 1994), but is supported by *in situ* hybridization to Dip-AST mRNA (Yu *et al.* 1995b). No physiological release of ASTs from these cells has been demonstrated, although Endo and Nishiitsutsuji-Uwo (1982) have produced images of exocytosis of secretory granules from putative endocrine cells of *P. americana*.

**Nerve fibres**

In addition to endocrine cells, AST immunoreactivity can be found in nerve fibres of the stomatogastric nervous system that extend over the surface of the midgut. These fibres form a grid pattern at the posterior and anterior ends of the midgut but are longitudinal throughout the mid-region (Reichwald *et al.* 1994). AST-immunoreactive fibres are absent from the most
anterior portion of the midgut (approximately one-quarter of its length) (Yu et al. 1995b). Although the nerve fibres in the midgut are rich in AST-immunoreactive material, Yu et al. (1995b) note that they do not hybridize with AST RNA probes. This suggests that the AST-like material within the nerve fibres is synthesized only in cell bodies and is transported through axons to target tissues. Some axons apposed to the outer muscle layer of the anterior midgut contain AST-immunoreactive neurosecretory granules, whereas others do not (Yu et al. 1995b).

According to Reichwald et al. (1994), the AST-immunoreactive nerve fibres in the midgut appear to be largely of stomatogastric origin. They can be traced through the esophageal nerve, recurrent nerve, and the frontal ganglion, possibly originating in one or two clusters of 6-10 cells in the tritocerebrum (Reichwald et al. 1994). In contrast, Yu et al. (1995b) argue that AST-immunoreactivity is localized to neurosecretory granules within the proctodeal innervation, and that this immunoreactivity can be attributed to peptide transport from cell bodies in the terminal abdominal ganglion, which express AST mRNA. In the cockroach B. germanica, the AST-immunoreactive esophageal nerve projects posteriorly from the frontal ganglion through the hypocerebral ganglion and into the foregut (Maestro et al. 1998). At the foregut, it divides into two gastric nerves, which run laterally to the proventriculus. The proventricular musculature is richly innervated by projections from the gastric nerves, which continue posteriorly into the midgut. The midgut in B. germanica is also innervated by branches of the proctodeal nerve and by a bilaterally symmetrical complex of associated small peripheral nerves, all of which show AST-immunoreactivity and which have their origin in the terminal abdominal ganglion.

The same AST gene is expressed in intrinsic endocrine cells of the midgut as in the central nervous system (Reichwald et al. 1994). Immunoassay of midgut tissues using antibodies raised against different members of the AST family suggests that the individual peptides appear to be
present in approximately equal amounts (Yu et al. 1995b). Reichwald et al. (1994) saw no
difference in the number of immunoreactive cells or their relative distribution using polyclonal
antibodies against two different members of the AST family. This evidence suggests that the
thirteen ASTs are probably released simultaneously from the precursor after processing (Yu et al.
1995b). However, it remains possible that different cell types will vary in their transcription and
post-translational processing of the AST gene (Reichwald et al. 1994).

Purpose of this study

The answers to many questions about ASTs will depend on a greater understanding of their
receptors. Putative ASTRs in the brain, CA, and hindgut of D. punctata have previously been
described and partially characterized (Cusson et al. 1991; Yu et al. 1995a). The abundance of
AST-immunoreactive endocrine cells and nerve fibres in the midgut (Reichwald et al. 1994; Yu
et al. 1995b), coupled with recent experiments showing an effect of ASTs on midgut enzyme
activities (Fusé et al. 1998), make the midgut a logical target for further examination of ASTRs.
The goals of the present study were to:

1) Prepare radioiodinated Dip-AST 7 of high specific activity;

2) Identify and characterize ASTRs in the midgut;

3) Evaluate how well different members of the AST family compete for binding
sites; and

4) Determine whether a synthetic pseudopeptide AST mimetic is able to compete
with natural ASTs for binding sites.

Determining a rank order of affinity for ASTRs in the midgut could have predictive value in
future midgut bioassays and could provide structure-activity clues that would lead to a greater
understanding of the ASTR binding site. This knowledge could be applied to the design of highly potent agonists or antagonists of ASTs with potential application as insecticidal agents.

2. Materials and methods

Animals

Insects (D. punctata) were reared as previously described (Szibbo & Tobe 1983). Mated females were collected on the day of emergence (day 0) and were maintained at 27°C on a 12:12 light:dark photoperiod, with food and water ad lib. until use.

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. Na\textsuperscript{125}I was purchased from Amersham (Arlington Heights, IL). Dip-ASTs were synthesized by Research Genetics or by the Core Facility of Insect Biotech Canada (Department of Biochemistry, Queen's University, Kingston, ON). The pseudopeptide Dip-AST analog AST(b)$2 was provided by R.J. Nachman (Food Animal Protection Research Laboratory, U.S. Department of Agriculture, College Station, TX).

Radioactivity measurement

All measurement of radioactivity was performed using a Beckman LS 6500 multi-purpose scintillation counter (Beckman Instruments Inc., Fullerton, CA) and CytoScint scintillation fluid (ICN Research Products Division, Costa Mesa, CA). The efficiency of this system for counting \textsuperscript{125}I-labelled samples is approximately 75%.
Preparation of $^{125}$I-Dip-AST 7

Dip-AST 7 was radioiodinated using Iodo-Beads (Pierce, Rockford, IL). One iodo-bead was washed twice with buffer (0.1 M sodium phosphate buffer, pH 6.5), then blotted dry on Whatman #10 filter paper. The bead was incubated for 5 minutes with Na$^{125}$I ($1.33 \times 10^5$ MBq [0.5 mCi] in 5 μl) and 100 μl of buffer, then 5.0 μg of AST (in 100 μl buffer) was added. The reaction was allowed to proceed for 7 minutes at room temperature (RT). The reaction was terminated by transferring the solution from the reaction vessel to a C$_{18}$ Sep-Pak (Waters Chromatography Division, Millipore Corporation, MA) containing 1.0 ml buffer, which had been equilibrated with 8 ml of methanol, 8 ml of H$_2$O, 8 ml of 0.1% trifluoroacetic acid (TFA), and 2 ml of 0.1% TFA containing 10 μg of bovine serum albumin (BSA). The Sep-Pak was washed with 5 ml 0.1% TFA, then with 5 ml 15% acetonitrile (ACN) containing 0.1% TFA. Finally, the labeled peptide was eluted with 2 ml 100% ACN containing 0.1% TFA.

Non-radioactive $^{127}$I-Dip-AST 7 was also prepared. Reaction conditions were identical, except that 50 μg of Dip-AST 7 was reacted with 55 mM Na$^{127}$I.

Purification of $^{125}$I-Dip-AST 7

Iodinated Dip-AST 7 was purified using reversed-phase HPLC. Aliquots of $^{125}$I-Dip-AST 7 in acetonitrile were concentrated in a Speed-Vac rotary vacuum concentrator (Savant) and resuspended in 250 μl 0.1% TFA. Samples were injected onto a 220 x 4.6 mm Vydac C$_{18}$ column (5 μm) connected to a Spectra-Physics SP8800 binary gradient pump with SP8500 dynamic mixer, SP8490 UV detector and SP4290 integrator/chart recorder. The column was equilibrated with a 5-minute wash with 0.1% TFA in water. A linear gradient of ACN + 0.1% TFA (0-45% in 45 min; 45-60% in 10 min; 60-100% in 10 min) at a flow rate of 0.5 ml/min was used to elute
peptides. The absorbance of the eluant was monitored at λ=280 nm. Fractions (0.25 ml) were collected during the gradient run. Aliquots (2 μl) of each fraction were assayed for incorporated radioactivity.

**Mass Spectrometry**

Mass spectrometry of selected HPLC peak fractions was performed at the Medical Sciences Mass Spec facility, University of Toronto, using a Perkin-Elmer Sciex API 3 triple-quadrupole electrospray mass spectrometer.

**Determination of specific radioactivity**

The specific radioactivity of labeled Dip-AST 7 was determined using a self-displacement radioimmunoassay (RIA) based on the methods of Herbert et al. (1965) and Stay et al. (1994). A standard curve was prepared by mixing known concentrations of “cold” unlabeled Dip-AST 7 with fixed amounts of labeled 125I-Dip-AST 7 and polyclonal antibody in 1.5 ml polypropylene microcentrifuge tubes. A control was prepared in which no unlabeled AST was present. All dilutions were made using RIA buffer (5.98 g Na2HPO4, 1.06 g KH2PO4, 1.50 g NaCl, 0.5 g protease-free BSA, and 0.05 g NaN3 in 500 ml H2O). The tubes were incubated overnight at 4°C, then free AST was separated from antibody-bound AST by the addition of dextran-coated charcoal (0.4 g Norit A charcoal (BDH Chemicals, Toronto, ON), 0.04 g dextran, in 50 ml RIA buffer). Charcoal plus free AST was pelleted by centrifugation and the quantity of bound AST was determined by counting the radioactivity in the supernatant. Results were plotted as a linear graph of B/B' against the quantity of “cold” AST used, where B = radioactivity bound by the control and B' = radioactivity bound in tubes with “cold” AST present. The quantity (ng) of
labeled $^{125}$I-Dip-AST 7 that had been added to each tube was determined as the point where the line intersected the abscissa by extrapolation (see figure 3 in results).

**Preparation of midgut plasma membranes**

Midguts (defined as the region of the alimentary canal between the gastric caeca and the Malpighian tubules) of day 1 to day 6 mated females were dissected in ice-cold Hepes buffer (10 mM Hepes, 5 mM MgCl$_2$, 1 mM PMSF, 1 mM EGTA and 1 mM 1,10-phenanthroline, pH 7.4). Midguts were cleaned of adhering fat body and tracheae and the peritrophic membrane and its contents were removed. The tissues were rinsed twice with Hepes buffer, then stored at $-70^\circ$C until a sufficient quantity had been collected for further processing. Tissues were homogenized in ice-cold Hepes buffer with a glass homogenizer. Homogenates were centrifuged twice (10 minutes x 1000 g, 4°C) to remove cellular debris, with supernatants collected and pellets re-homogenized after each spin. Pooled supernatants were then centrifuged at high speed (35 minutes x 35000 g, 4°C) to form pellets that contained plasma membranes. Pellets were washed once with Hepes buffer, resuspended in Hepes buffer and finally adjusted to a concentration of 1 μg protein / μl. Total protein was determined by the Bio-Rad standard protein assay using 0.1% Triton X-100 as detergent and BSA as protein standard. Aliquots of midgut plasma membranes were then stored at $-70^\circ$C until use.

**Binding assay**

Binding assays were carried out in 1.5-ml polypropylene microcentrifuge tubes according to the method of Yu et al. (1995a). Typically, 20 μg of membrane preparation was incubated with appropriate amounts of radioiodinated AST for 60 minutes at RT in a final volume of 250 μl of binding buffer (Hepes buffer containing 15 mg/ml protease-free BSA, pH 7.4, with one
Complete™ Mini protease inhibitor cocktail tablet (Boehringer Mannheim) per 10 mL) at room temperature. Incubations were terminated by centrifugation (35 minutes x 35000 g, 4°C). Aliquots of the supernatant were counted to determine the concentration of free ligand in each incubation. Remaining supernatant was aspirated and discarded. Pellets were washed with 600 μl ice-cold Hepes buffer, then collected by cutting the tips of the microcentrifuge tubes just above the pellets. Pellets were counted to determine the quantity of bound ligand. Results were analyzed using Graphpad Prism software (Version 2.01 for Windows 95, GraphPad Software, Inc., San Diego, CA).

**Saturation binding**

Midgut plasma membranes were incubated with increasing concentration of ¹²⁵I-Dip-AST 7 (from 0.1 to 15 nM) in the presence (non-specific binding) or absence (total binding) of 2.5 μM unlabeled Dip-AST 7. Specific binding was calculated by subtracting non-specific binding from total binding. Saturation binding curves were analyzed using a nonlinear regression model for

$$B_L = \frac{B_{max}[L]}{K_D + [L]} \quad \text{(equation 1)}$$

$$B_L = \frac{B_{max1}[L]}{K_{D1} + [L]} + \frac{B_{max2}[L]}{K_{D2} + [L]} \quad \text{(equation 2)}$$

one site binding (equation 1) or two site binding (equation 2).

**Reversibility**

Midgut plasma membranes were incubated with 10 nM ¹²⁵I-Dip-AST 7. After 60 minutes, 20 nM non-radioactive ¹²⁷I-Dip-AST 7 was added to half of the replicate tubes while the other half were processed to determine control specific binding (as above). After a further 60 minutes, the specific binding in the remaining tubes was determined (as above).
**Competitive binding assays**

Midgut plasma membranes were incubated with 10 nM $^{125}$I-Dip-AST 7 and 10 nM, 100 nM, or 500 nM unlabeled competitor (competitors included Dip-ASTs 2, 5, 7, 9, 11, and 13, as well as the pseudopeptide Dip-AST analog AST(b)$\phi$2). Specific binding of $^{125}$I-Dip-AST 7 was determined after 60 min (as above).

3. Results

*Preparation, purification and identification of $^{125}$I mono-iodinated allatostatin*

Dip-AST 7 contains a single tyrosine residue that can serve as a potential iodination site. A single tyrosine residue can accommodate one or two iodine atoms. Mono-iodinated AST was separated from unlabeled and di-iodinated AST by reverse-phase HPLC. Four major peaks (figure 1, upper panel, peaks ‘A’, ‘B’, ‘C’, ‘D’) and two minor peaks (peaks ‘E’ and ‘F’) were seen following separation of the products of a non-radioactive iodination procedure, using $^{127}$I. Peak ‘B’ was identified as unreacted, native Dip-AST 7 on the basis of retention time. Iodine is a bulky, hydrophobic group and typically increases the retention time of labeled peptides on reverse-phase HPLC columns. Peaks ‘C’ and ‘D’ eluted later than the retention time of unlabeled standard Dip-AST 7. A second iodination was performed using radiolabeled iodine ($^{125}$I). HPLC fractions were collected and assayed for incorporated radioactivity (figure 1, lower panel). One major and one minor radioactive peak was identified. The major radioactive peak had the same retention time as peak ‘C’ from the non-radioactive HPLC run. The minor radioactive peak co-eluted with peak ‘F’ from the non-radioactive iodination.
Figure 1. HPLC separation of Dip-AST 7 iodination products.

*Upper panel:* HPLC profile of reaction products following iodination of Dip-AST 7 with "cold" Na\textsuperscript{127}I. For this reaction, atypically high concentrations of NaI and peptide were employed to produce a clear chromatographic trace and to obtain sufficient product for further analysis. Product peaks are identified in the text as 'A', 'B', 'C' etc. Peak 'B' has retention time identical to native Dip-AST 7. *Lower panel:* Incorporation of radioactive \textsuperscript{125}I into products of a "hot" iodination reaction was monitored by scintillation counting of sequential fractions from the HPLC elution volume. Two distinct peaks of radioactivity had retention times matching those of peaks 'C' and 'F' from the "cold" iodination. Peak 'C' was tentatively identified as mono-iodo Dip-AST 7 on this basis.
On the basis of the incorporated radioactivity, peak ‘C’ was tentatively identified as mono-iodo Dip-AST 7. To verify that this product carried only a single iodine atom, a sample of peak ‘C’ was analyzed by electrospray ionization mass spectrometry (ESI-MS) (figure 2). The theoretical mass for Dip-AST 7 carrying a single $^{127}$I label is 1460 Da. The final estimated mass of the sample fraction was 1460.59 ± 0.28 Da.

**Specific radioactivity of mono-iodo Dip-AST 7**

In order to correctly calculate the quantity of AST binding to putative receptors, it was necessary to determine the amount of radioactivity “carried” by each unit of labeled AST. Theoretically, a mono-iodinated peptide should have the same specific activity as $^{125}$I, *i.e.* 2.9 x $10^5$ MBq/nmol [2190 Ci/mmol]. The self-displacement radioimmunoassay method of Herbert *et al.* (1965) was used to determine the specific activity of mono-iodinated Dip-AST 7 (figure 3). We found that a typical iodination produced mono-iodinated Dip-AST 7 with a specific activity of $1.33 \times 10^4$ – $1.2 \times 10^5$ MBq/nmol [100 – 900 Ci/mmol].

**Allatostatin binding in the midgut**

Mono-iodinated Dip-AST 7 appeared to bind to midgut membrane preparations. Some of the bound AST was not displaced by excess, non-iodinated AST and was therefore considered non-specific binding. Non-specific binding increased in a linear fashion when membranes were incubated with increasing concentrations of radiolabeled AST (data not shown) and did not appear to be saturable. Specific binding made up only a small proportion (~20-30%) of total
Figure 2. Ion-electrospray mass spectrometry.

The identity of an iodination product that was believed to be mono-iodinated Dip-AST 7 was confirmed by determination of relative mass. Ionization produced two ions; the 2+ ion (A) had a relative mass of 730 Da and the singly-charged ion (B) had a relative mass of 1461 Da. Average mass of the parent compound was calculated to be 1461 ± 0.28 Da. Other peaks for which a mass estimate could be obtained did not correspond to the mass of either Dip-AST 7 or any iodinated AST product.
Figure 3. Quantitation of specific radioactivity by self-displacement RIA.

The specific radioactivity of mono-iodinated Dip-AST 7 was quantified using a self-displacement RIA procedure. Radioactivity of Ab-bound AST was expressed as a linear graph of B/B' against the quantity of unlabeled AST in each incubation, where B = radioactivity bound in the absence of unlabeled AST and B' = radioactivity bound with unlabeled AST present. Positive values on the abscissa (X-axis) represent the quantity (ng) of unlabeled AST in each incubation, whereas negative values represent the fixed quantity of $^{125}$I-Dip-AST 7 added to each incubation. In this example ($n=3$), the extrapolated line intersects the abscissa at -0.28, indicating that 0.28 ng of radiolabeled AST was added. This amount of radiolabeled AST produced 33421 cpm in the scintillation counter; therefore, the specific activity is 33421 cpm/0.28 ng or $1.5 \times 10^4$ MBq/nmol [112 Ci/mmol].
binding but was well above background. Ligand depletion (change in incubation ligand concentration caused by specific binding to receptors) was less than 15% (data not shown).

Specific binding at first increased proportionately with increasing low concentrations of radiolabel, but showed evidence of saturation as the concentration of radiolabel increased (figure 4). Nonlinear regression analysis of the binding isotherm indicated that a one-site binding model fit the data better than a two-site model. According to the model, the binding sites are of low abundance \( B_{\text{max}} = 1.5 \pm 0.1 \) pmol/mg protein and moderate affinity \( K_d = 14.0 \pm 2.2 \) nM.

Reversibility

To provide further evidence that the binding of AST to midgut cell membranes represented a receptor-ligand interaction, it was necessary to demonstrate that the specific binding of \(^{125}\)I-Dip-AST 7 to midgut cell membranes was reversible. \(^{125}\)I-Dip-AST 7 was allowed to reach a steady state with midgut cell membranes, and we then attempted to displace AST from binding sites by adding excess non-radioactive \(^{127}\)I-Dip-AST 7. We observed that 20 nM of \(^{127}\)I-Dip-AST 7 was able to displace approximately 40% of the radiolabel that had been specifically bound (figure 5). This indicated that the specific binding of Dip-AST 7 to binding sites was a reversible (non-covalent) phenomenon.

Competitive potency of selected ASTs

We elected to use a single-point competitive binding assay to quickly screen different members of the Dip-AST peptide family for binding in the midgut. In this assay, a single concentration of non-radioactive "competitor" AST is incubated with midgut cell membranes in combination with a single concentration of \(^{125}\)I-Dip-AST 7. After 60 minutes, the amount of
Figure 4. Saturable binding to midgut plasma membrane by Dip-AST 7.

Midgut plasma membranes (20 μg) were incubated for 60 min at RT with increasing concentrations of $^{125}$I-Dip-AST 7 in the absence (total binding) or presence (non-specific binding) of 2.5 μM unlabeled Dip-AST 7. Specific binding was calculated by subtracting nonspecific binding from total binding. Individual data points from five separate experiments were pooled, and curve fitting was performed with Graphpad Prism software using nonlinear regression.
Figure 5. Reversibility of Dip-AST 7 binding.

Steady-state binding of 10 nM $^{125}$I-Dip-AST 7 (t=60 min, n=4) could be partially reversed by the addition of excess (20 nM) "cold" $^{125}$I-Dip-AST 7 (t=120 min, n=4). Binding at t=120 min is expressed as a percentage of control binding at t=60 min.
$^{125}$I-Dip-AST 7 Binding
% of control

$t = 60$ min

$t = 120$ min

20 nM "cold" $^{127}$I-Dip-AST 7 added at $t=60$ min
radiolabeled AST bound in tubes containing a competitor is compared to the amount bound in the absence of competitor. We found that low concentrations (10 nM) of different members of the Dip-AST peptide family displaced different amounts of $^{125}$I-Dip-AST 7 (figure 6). Statistical analysis (by $t$ test) revealed that the apparent differences were not significant at the 95% level of confidence, probably due to high variability in the data (SEMs ranged from 10-20%). At a less stringent confidence level (90%), both Dip-AST 2 and Dip-AST 7 produced significant levels of displacement ($P=0.091$ and 0.078, respectively). We were more successful using a higher concentration of competitors (500 nM). At this concentration, relative differences between different Dip-ASTs were more apparent, and variability in the data was lower. All of the Dip-ASTs tested, with the exception of Dip-AST 13, were able to displace significant amounts of radiolabeled Dip-AST 7 ($P<0.05$; figure 6). Unlabeled Dip-AST 7 and Dip-AST 2 were the best competitors. Dip-AST 7 completely displaced the radiolabeled AST. The difference in displacement between Dip-AST 2 and Dip-AST 7 was not significant ($P=0.0634$). Dip-ASTs 5, 9, and 11 all displaced less radiolabeled AST than Dip-AST 7 ($P<0.005$) and Dip-AST 2 (except Dip-AST 9, $P=0.054$), but could not be distinguished from one another statistically.

*Competitive potency of a pseudopeptide analog*

The single-point competitive displacement assay was used again to assess the AST pseudopeptide analog $\text{AST(b)}\phi 2$ as a competitor against native ASTs. We wished to determine whether this modified analog was still able to interact with AST binding sites. The analog (100 nM) was able to displace approximately 40% of the radiolabeled Dip-AST 7 (figure
Figure 6. Competitive potency of selected Dip-ASTs.

A single-point competitive binding strategy was used to determine the competitive potency of selected Dip-ASTs. Midgut plasma membranes (20 μg) were incubated with 10 nM $^{125}$I-Dip-AST 7 and either 10 nM (panel A) or 500 nM (panel B) unlabeled competitors. Specific binding of $^{125}$I-Dip-AST 7 was measured after 60 minutes. Control levels of specific binding were measured in the absence of competitors. % Displacement was calculated by subtracting specific binding in the presence of competitor from control specific binding, and expressing the difference as a fraction of control specific binding. Results of statistical comparisons are described in the text. (For panel A, $n=7$-$8$ for experimental values and $n=12$ for control; for B, $n=3$ for experimental values and $n=6$ for control. ND= not determined.)
Figure 7. Competitive potency of a pseudopeptide Dip-AST analog

Midgut plasma membranes were incubated with 10 nM $^{125}$I-Dip-AST 7 in combination with 100 nM unlabeled Dip-AST 2 ($n=6$) or 100 nM of the pseudopeptide Dip-AST analog AST(b)$\phi$2 ($n=4$). Specific binding of $^{125}$I-Dip-AST 7 was measured after 60 minutes. Control specific binding ($n=6$) was measured in the absence of competitors. % Displacement was calculated by subtracting specific binding in the presence of competitor from control specific binding, and expressing the difference as a fraction of control specific binding.
7), but variability was high and this displacement was not significant by t test (P=0.16). In comparison, 100 nM Dip-AST 2 displaced approximately 50% of the radiolabeled AST, and this displacement was significant (P < 0.05).

4. Discussion

Preparation and purification of radiolabeled Dip-AST 7

The preparation of the radiolabeled ligand is one of the most important aspects of a radioligand-binding assay (Limbird 1996). We chose to label Dip-AST 7 with $^{125}$I, because its high specific radioactivity allows detection of binding sites from small quantities of tissue. This is particularly important when evaluating receptors in insects, because dissection of large quantities of insect tissues is time-consuming. Preparing iodinated peptides is a relatively simple procedure. Iodine will readily participate in a nucleophilic attack on tyrosine residues in the presence of an oxidizer (Hunter and Greenwood 1962). Nearly all of the ASTs, including Dip-AST 7, possess a tyrosine residue and are good candidates for iodination. We used Iodo-Beads™, which in our hands produced more consistent results than the commonly used Chloramine T procedure (Hunter and Greenwood 1962). The oxidative component of Iodo-Beads™ is covalently bound to small polystyrene beads, which allows rapid termination of the oxidative reaction and has the added advantage of reducing oxidative damage to the peptide (Markwell 1982). The products of an iodination reaction include unreacted peptide, in addition to labeled peptides that may contain one or two iodine atoms in each tyrosine ring. We used HPLC to separate mono-iodinated Dip-AST 7 from other products of the iodination reaction. Purifying $^{125}$I-Dip-AST 7 in this manner increases the specific radioactivity of the ligand, thus improving the sensitivity of the binding assay (Seidah et al. 1980). It also removes the confounding binding
effects of the di-iodinated peptide, which is likely to have a different affinity for the receptor (Kurcibart et al. 1971; Radicella et al. 1985; Tsomides and Eisen 1993). Examination of the HPLC traces and profiles of incorporated radioactivity indicated that the iodination reaction appears to greatly favour the formation of mono-iodinated product over di-iodinated product. Steric hindrance may impede the approach of a second iodine atom. Elution time increased with each iodine atom incorporated, as we had predicted based on the increased hydrophobicity imparted by each iodine group (Seidah et al. 1980). Several product peaks identified by HPLC did not appear to correspond to radiolabeled AST. In particular, one product eluted several minutes earlier than native Dip-AST 7 (figure 1, upper panel, peak ‘A’). Seidah et al. (1980) found that peptides that contained Met residues shifted to earlier elution volumes during reverse-phase HPLC when they were deliberately oxidized. Amino acid side chains that have been demonstrated to be vulnerable to oxidation include Met, Cys, and Trp (Tsomides and Eisen 1993). Dip-AST 7 does not contain any of these residues, but it is nevertheless plausible that the early-eluting product peak could represent AST damaged by oxidation. The identity of this peak is not of particular interest, since it does not interfere with the recovery of radiolabeled AST. However, it could potentially be identified by examining the HPLC profile of deliberately oxidized Dip-AST 7.

From binding site to receptor

We have demonstrated that the midgut of adult female D. punctata contains a single class of binding sites for Dip-AST 7. Do these binding sites truly represent AST receptors (ASTRs)? There are no biochemical means to ascertain that a binding site truly corresponds to a receptor site (Laduron 1984). Many binding sites identified by radioligand-binding assays may be
completely unrelated to physiological receptors (Laduron 1984; Kenakin et al. 1992). According to Cooper et al. (1996), authentic receptors should be saturable, specific, and reversible. Receptor binding is typically characterized by a high affinity for the ligand and a low capacity. We have demonstrated that binding sites for Dip-AST 7 in the midgut have a low capacity (~1.5 pmol/mg protein) and saturate at nanomolar concentrations of AST (Kₜ=14 nM). Specificity was demonstrated by the differential ability of structurally similar members of the AST peptide family to displace binding. We showed that the binding of radiolabeled Dip-AST 7 was a reversible process; the addition of excess unlabeled AST produced a new binding equilibrium and displaced radiolabeled AST that had previously been bound. In addition to physiological criteria, modern receptor biology demands further evidence before a binding site can be accepted as a receptor. Once the components of a putative receptor system have been identified, it should be possible to isolate and purify them, and then reconstitute them to restore activity. This requirement, which Cooper et al. (1996) have referred to as “putting Humpty-Dumpty back together again”, has rarely been satisfied even for the most well-characterized receptors. The molecular characterization and cloning of the receptor gene followed by its successful expression in a well-defined system serves as the ultimate identification. Until we have met this final criterion, we will continue to refer to the AST binding site in the midgut as a “putative” receptor.

Correlation of affinity to biological activity

An essential element in defining a binding site as a receptor is the correlation of affinity with biological activity (Laduron 1984). Fusé et al. (1998) have found that isolated midguts incubated with Dip-AST 7 contain greater invertase and α-amylase activity in the lumen than control
tissues. This response to Dip-AST 7 is dose-dependent and has an EC$_{50}$ in the low nanomolar range, approximately equivalent to the affinity of radiolabeled Dip-AST 7 for midgut binding sites. Data on the potency of other ASTs in this assay are still forthcoming. If the binding site we have demonstrated does represent a receptor, then we might predict the rank order of potency to follow our estimated rank order of affinity: Dip-AST 7 > Dip-AST 2 > Dip-AST 5, 9, 11 > Dip-AST 13. However, this prediction will only prove accurate if all of the ASTs have full intrinsic activity. In addition, some of the ASTs may bind to other receptors that were not detected by $^{125}$I-Dip-AST 7.

The affinity of the midgut binding sites is approximately tenfold lower than the affinity of the putative receptors that have been described in the brain and the CA (Yu et al. 1995a). AST concentration in the haemolymph of D. punctata has been shown to be between 0.1 and 2.4 nM (Yu et al. 1993; Woodhead et al. 1993). The local concentration may be much higher close to endocrine release sites, such as the AST-immunoreactive putative endocrine cells in the midgut (Yu et al. 1995b). Using a rearranged form of the Langmuir binding isotherm equation (equation 3), it is possible to calculate that the occupancy of the putative midgut receptors would vary from 0.7% to 14% as the haemolymph AST concentration varies from 0.1 nM to 2.4 nM. However, the affinity of the putative receptor for $^{125}$I-Dip-AST 7 may not accurately reflect the affinity of unlabeled Dip-AST 7, which is presumably the endogenous ligand. The addition of an iodo group adds bulk and hydrophobicity (Kurcbart et al. 1971; Radicella et al. 1985) which may affect the interaction of the ligand with the receptor. Yu et al. (1995a) demonstrated that mono-
iodinated Dip-AST 7 retains high potency as an inhibitor of JH biosynthesis, which suggests that the interaction of Dip-AST 7 with its receptor in the CA is not greatly disrupted by the additional iodine atom.

Reversibility

In our reversibility assay, we established an initial binding steady state with 10 nM $^{125}$I-Dip-AST 7. According to our estimates of $K_D$ and $B_{max}$, this concentration will result in an occupancy of 41.6% of the putative ASTRs. Next, we added an additional 20 nM of $^{127}$I-Dip-AST 7, bringing the total ligand concentration to 30 nM. At this concentration, 68.2% of binding sites should be occupied. There is no structural difference between $^{125}$I-Dip-AST 7 and $^{127}$I-Dip-AST 7, and accordingly, these compounds should display equal affinity for binding sites. Because only one-third of the I-Dip-AST 7 present at the new equilibrium was radiolabeled, we predicted that one-third of the occupied receptors (22.7% of the total receptors) would be radiolabeled. Therefore, the radioactive signal should decrease by 45.5%. In fact, we found that the addition of 20 nM $^{127}$I-Dip-AST 7 decreased the radioactive signal by 40±10%, in good agreement with our prediction. This experiment thus not only demonstrates that the binding of ASTs to the putative receptor is a reversible phenomenon, but also supports our estimates of $K_D$ and $B_{max}$.

Abundance of the midgut receptor and consequences for purification

The putative midgut receptors appear to have similar capacity to those in the CA, ~1.5 pmol/mg protein. Because we found only a single class of binding site, this capacity likely also represents the abundance of receptors within the tissue. A typical Diploptera midgut provides approximately 0.01 mg of cell membranes after processing, which would represent 15 fmol of receptors. SDS-PAGE of photoaffinity labeled AST receptors suggests that they have a relative
molecular mass of approximately 40,000 (Cusson et al. 1991; Yu et al. 1995a). Thus, a single midgut could theoretically provide up to 0.6 ng of purified receptor protein, assuming no loss during isolation. It is our hope that a molecular approach to sequencing the receptor will prove fruitful, obviating the need for protein isolation. However, even a partial amino acid sequence could permit the design of a cDNA probe, which would facilitate the cloning of the gene.

Receptor modulation

Our results indicate that only a single class of binding site is responsive to Dip-AST 7 within the range of concentrations we tested. Because our experiments utilized midguts from cockroaches from one to six days old, this result indicates that there is likely no modulation of receptor affinity during the first six days of adult life. Any such modulation would have appeared as a second class of binding sites during analysis of the saturation binding curve. It is possible that modulation of receptors could occur later in the reproductive cycle. Yu et al. (1995b) reported that the levels of AST-immunoreactive material found in the gut of Diploptera change during the reproductive cycle, following a pattern similar to the changes of AST expression in brain tissue. Dip-AST 7-immunoreactivity in the midgut is essentially constant from day 0 to day 5 of the cycle, and begins to increase significantly at day 6 (Yu et al. 1995b). Evidence of a change in receptor abundance or affinity might have been apparent in midguts from older animals. Dip-AST 7-immunoreactivity in the midgut appears to reach a maximum at day 7 (Yu et al. 1995b). Comparing the properties of receptors prepared from midguts of day 7 animals to those of younger cockroaches might provide valuable information regarding the regulation of ASTRs. It could also be instructive to examine the abundance of binding sites in the midgut immediately after a larval molt. In the cockroach B. germanica, the epithelium of the midgut is
regenerated during each molt (Weyer 1936, cited in Wigglesworth 1974). If this is also true in Diploptera, it might be possible to follow the appearance of receptors in the newly deposited epithelium.

Multiple receptor subtypes

It is possible that the midgut contains one or more additional classes or subtypes of AST receptors not detected by our assay. These receptors might have no affinity for Dip-AST 7, or an affinity that is far outside the range of concentrations we employed. The inability of Dip-AST 13 to displace significant amounts of radiolabeled Dip-AST 7 supports the possibility that a second class of receptors might recognize other Dip-ASTs. This other receptor, if it exists, must have very low affinity (if any) for Dip-AST 7, or it would have appeared as a second class of binding sites during analysis of the saturation binding isotherm. Dip-AST 13 is unique among the cockroach ASTs because it possesses a C-terminal Ile-NH₂ residue, rather than the typical Leu-NH₂. Hayes et al. (1994) showed that the C-terminal Leu of Dip-AST 5 was the most important single amino acid side chain for retention of potency as an inhibitor of JH biosynthesis. It has been suggested that this side chain plays an important role in the binding and/or activation of target cell receptors. As might be expected, Dip-AST 13 has low potency as an inhibitor of JH biosynthesis, presumably because it is relatively ineffective at binding to or activating receptors in the CA. In the hindgut, however, Dip-AST 13 is among the most potent inhibitors of muscle contraction, ranking 2nd of the thirteen Diploptera ASTs (Lange et al. 1995), suggesting that there are receptors that effectively recognize this peptide. It will be of value to determine the potency of Dip-AST 13 as a modulator of midgut enzyme activity. If it displays significant biological activity, it will almost certainly indicate that a second class or subtype of ASTR exists
Figure 8. Comparison of standard and single-point competitive binding assays.

In panel A, the potency of different unlabeled competitors x, y, and z is determined by calculating their IC$_{50}$ values, i.e. the concentration of competitor that reduces specific radioligand binding to 50% of control levels. In panel B, the relative potency of each competitor is determined by the amount of radioligand that is displaced at a single concentration (arrow). Note that the low-potency competitor z would not appear to compete for binding at all at this concentration. Panel A is based upon a figure in Limbird (1996).
in the midgut. It will also be instructive to radioiodinate Dip-AST 13 and measure its binding parameters in the midgut, and to determine whether Dip-AST 7 can displace it.

*Limitations of the single-point competitive binding assay*

The specificity of the interaction between a ligand and its receptor has classically been studied with competitive binding experiments. In these experiments, receptor preparations are incubated with a single concentration of a radioligand in the presence of increasing concentrations of unlabeled competitors (Limbird 1996). The order of potency of the competing agents can then be determined by examining the competition profiles, and determining the concentration of competitor required to displace 50% of control radioligand binding (IC$_{50}$). Under the appropriate conditions, it is possible to determine the absolute affinity (K$_d$) of each competitor from the IC$_{50}$ (Cheng and Prusoff 1973, and Chou 1974, cited in Limbird 1996). It is sometimes also possible to determine the complexity of the receptor-competitor interaction by assessing the steepness of the competition profile (Limbird 1996). In contrast, the single-point competitive binding assay is strictly limited to estimating the relative potency of a series of unlabeled agents in competing for radioligand binding. As in the more traditional assay, the concentration of radioligand is fixed. Rather than assaying the effects of competitors over a range of concentration, each competing agent is assayed at a single concentration, which would normally be a single point on a competition profile. The relative potency of different competitors can be readily determined, because a more potent agent will displace more radioligand than a poorer competitor at a given concentration (figure 8). However, it is not possible to determine an absolute measure of potency from such an assay, nor is it possible to determine if the receptor-competitor interaction obeys the law of mass-action or a more complex binding model.
Furthermore, depending on the concentration of competitor that is chosen, it may be difficult to distinguish differences in potency between different compounds. Despite its disadvantages, the single-point competitive binding assay is a valuable tool. Producing a full competition profile for a large number of competing agents consumes a prohibitively large amount of tissue. This is not a particular concern in the study of receptors in larger animals, as large amounts of receptor preparation can be obtained with minimal time invested in dissection. Studying receptors in insect tissues, however, may require long hours of microdissection to obtain relatively small amounts of plasma membrane. The single-point assay allows a large library of potential competitors to be screened with a minimal investment of time and tissue. Time can then be spent more profitably studying the most potent competitors in detail.

*Relationships between AST structure and competitive potency*

We observed that the ASTs used in the single-point competition assay could be separated into three groups, having high (Dip-AST 7, Dip-AST 2), moderate (Dip-AST 5, Dip-AST 9, Dip-AST 11), or low (Dip-AST 13) potency as competitors for the Dip-AST 7 binding site (table 1). Do the members of each group have common structural characteristics that might explain their similar affinity for the putative receptor? Dip-AST 2 and Dip-AST 7 were the most potent competitors. Dip-AST 2, an octadecapeptide, is the longest AST, and Dip-AST 7, a tridecapeptide, is the third longest. Length of the N-terminal “address” sequence may be a significant factor in AST binding to midgut receptors, with additional residues serving to stabilize the receptor-ligand interaction. We have not yet tested Dip-AST 10, the second longest
Table 1. Relative competitive potency of ASTs for midgut binding sites.

Potency of ASTs was classified as high, moderate, or low based on the results of a single-point competitive binding assay (see figure 7). The amino acid sequence motif Arg-Leu (underlined) may be involved in the receptor-ligand interaction (see text).
<table>
<thead>
<tr>
<th>Dip-AST</th>
<th>Amino Acid Sequence</th>
<th>Competitive potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>APSGAQRLYGFGL-NH$_2$</td>
<td>High</td>
</tr>
<tr>
<td>2</td>
<td>AYSYVSEYQRLPVYNFGL-NH$_2$</td>
<td>High</td>
</tr>
<tr>
<td>5</td>
<td>DRLYSFGL-NH$_2$</td>
<td>Moderate</td>
</tr>
<tr>
<td>9</td>
<td>GDGLYAFGL-NH$_2$</td>
<td>Moderate</td>
</tr>
<tr>
<td>11</td>
<td>YPQEHRSFGL-NH$_2$</td>
<td>Moderate</td>
</tr>
<tr>
<td>13</td>
<td>IPMYDFGI-NH$_2$</td>
<td>Low</td>
</tr>
</tbody>
</table>
AST, as a competitor. Dip-AST 11, which is the fourth longest AST, displayed only moderate potency as a competitor for binding, suggesting that extra stability requires a certain minimum length, or that particular residues rather than length alone, are required within the N-terminal sequence. However, it is important to note that bioassays of in vitro JH biosynthesis by CA do not strongly support length of the N-terminal sequence as a primary factor in biological activity (Stay et al. 1994; Tobe et al. 1994). Dip-AST 2 and Dip-AST 7 also share an Arg-Leu motif close to the C-terminal pentapeptide. This motif directly adjoins the C-terminal pentapeptide in the Dip-AST 7 sequence, but is separated by Pro-Val in the Dip-AST 2 sequence. It is perhaps significant that the Arg-Leu site is a target for proteolytic cleavage by peptidases associated with both haemolymph and tissue homogenates (Garside 1997a,b). If this motif is important for conferring increased affinity, then its cleavage would be expected to reduce potency. In fact, such cleavage products do show greatly reduced potency as inhibitors of JH biosynthesis (Pratt et al. 1991a; Stay et al. 1991b; Veelaert et al. 1996). The significance of the Arg-Leu motif for high-affinity binding is not clear, because it is also shared by Dip-AST 5 and Dip-AST 9, which are only moderately potent competitors. A combination of structural motifs may be required for full potency. Dip-AST 5 and Dip-AST 9 also share an Asp residue close to the Arg-Leu motif. This residue is flanked by Gly in the Dip-AST 9 sequence, but Gly is a small amino acid without bulky side chains and may not prevent Asp from interacting with an appropriate receptor domain. It is difficult to correlate the competitive potency of Dip-AST 11 with structural motifs. The N-terminal sequence of Dip-AST 11 bears little similarity to those of ASTs with similar competitive potency. Even the C-terminal pentapeptide of Dip-AST 11 features a Phe residue in the −5 position, rather than the more common Tyr.
As mentioned previously, Dip-AST 13 is unique among the Diploptera ASTs in having an Ile residue in place of the C-terminal Leu. This substitution may greatly reduce the binding affinity of this peptide. However, Pratt et al. (1991a) have reported that a synthetic variant of Dip-AST 2 featuring a C-terminal Ile residue has similar potency to the authentic peptide as an inhibitor of in vitro JH biosynthesis. It may be that additional motifs in the N-terminal sequence of Dip-AST 2 confer stability to the receptor-ligand complex even in the absence of the C-terminal Leu, or that a second subtype of ASTR recognizes ASTs with a C-terminal Ile. Interestingly, the structure of the Diploptera AST gene predicts two additional peptides with C-terminal Ile residues (Donly et al. 1993). These peptides have not been studied extensively because they are non-amidated, and therefore presumably ineffective as inhibitors of JH biosynthesis (Pratt et al. 1989, 1991b; Duve et al. 1993). A C-terminal amide also appears to be essential for modulation of hindgut motility (Lange et al. 1995).

It is important to note that none of the experiments which identified the core sequence of ASTs employed midgut tissue. There is therefore no direct evidence that the C-terminal pentapeptide is responsible for binding to ASTRs in the midgut. However, if the C-terminal pentapeptide is ignored, the remaining N-terminal sequences are highly variable and bear little resemblance to one another. It is difficult to understand how Dip-AST 11, for example, could displace Dip-AST 7 if the key sequence for binding is located within the N-terminal, as the N-terminal sequences of these peptides are almost completely dissimilar. It is more reasonable to assume that the highly conserved C-terminal is involved in the receptor-ligand interaction. Until it is proven otherwise, however, we should not assume that this interaction requires the entire C-terminal pentapeptide. Further experiments using both C-terminally truncated and internally
substituted synthetic ASTs (as in Hayes 1994) will ultimately be required to identify the core sequence of ASTs with respect to midgut receptors.

Agonism and partial agonism

Traditionally, receptors have been classified by the relative potencies and selectivities of their agonists and antagonists (Kenakin et al. 1992). Agonists are compounds that bind to and activate receptors, whereas antagonists interact with the receptor recognition site but fail to initiate intracellular signal transduction. At present, there are no proven antagonists of ASTRs. Recently, however, a peptide identical to Dip-AST 2^{11-18} isolated from the locust Schistocerca gregaria has been shown to have no inhibitory activity on the CA of 2-day-old virgin female D. punctata, and in fact appears to slightly stimulate JH biosynthesis in these animals. It has been proposed that this peptide fragment is an antagonist of the ASTR and that apparent stimulation is the result of endogenous ASTs being blocked from the receptor (Veelaert et al. 1996). In the radiochemical assay for JH biosynthesis, each of the thirteen different Dip-ASTs have different potency, but all show full efficacy (produce the maximal effect) if they are applied at sufficiently high concentrations (Stay et al. 1994). To date, the default assumption appears to have been that all of the ASTs have equal intrinsic activity and are full agonists of ASTRs. All full agonists will produce the same level of effect at equal levels of receptor occupancy (Matthews 1993). In this case, a rank order of potency will solely reflect differences in the affinity of the compounds for the receptor. However, it is possible that some of the Dip-ASTs are only partial agonists, with less than maximal intrinsic activity. Full and partial agonists can both display full efficacy (as in the JH radiochemical assay) if spare receptors are present, because only a small fraction of the receptors must be occupied for full activation of signal transduction mechanisms. Spare receptors
permit a response to low concentrations of agonists that have low affinity for the receptor, because full occupancy is not required to produce a significant effect. Agonists with low affinity have a rapid rate of dissociation, which has the advantage of allowing swift termination of signal transduction (Limbird 1996). A rank order of potency of partial agonists will reflect not only differences in affinity, but also differences in the intrinsic activity of each ligand. It is therefore possible that the rank order of affinity we have determined for the putative midgut ASTR may not reflect the rank order of potency of the ASTs in a bioassay, such as the enzyme activity bioassay. It should be possible (although not trivial) to determine whether different members of the Dip-AST family have equal intrinsic activity once their affinity for a given receptor has been determined. When the affinity is known, it is simple to calculate the concentration that will produce a given proportion of receptor occupancy. Then, one would apply the ASTs in a well-characterized bioassay and determine whether equal levels of receptor occupancy produce equal effects.

It will be instructive to determine if different ASTs have different intrinsic activity, because partial agonists can act as competitive antagonists of full agonists. The Dip-ASTs are the product of a single gene (Donly et al. 1993) and may well be processed and released simultaneously (Tobe et al. 1994). If this is the case, ASTs with high affinity but low intrinsic activity could potentially block access of ASTs with high intrinsic activity but lower affinity from receptor binding sites. Lower-affinity ASTs may depend on proteolytic enzymes, which degrade ASTs in the haemolymph (Garside et al. 1997a) and on target tissues (Garside et al. 1997b) to selectively degrade less active partial agonists and allow them access to receptor sites. Alternately, the metabolism of ASTs could halt their biological activity in two ways: first, by reducing the concentration of agonists near binding sites, and second, by blocking binding sites if the products
of metabolism are themselves partial agonists with high affinity for the receptors. It has been established that some of the intermediate products of AST metabolism are able to inhibit JH biosynthesis in the CA, albeit with greatly reduced potency (Garside et al. 1997b). However, the affinity of Dip-AST 5 metabolic products for binding sites in brain membranes is much lower than that of the full peptide (Yu, Bendena and Tobe, unpublished). This suggests that products of AST metabolism probably do not interfere with the binding of intact ASTs.

*Protease activity in the midgut*

Previous attempts to characterize ASTRs in the midgut of *Diploptera* were unsuccessful (Yu, Bendena and Tobe, unpublished data). These experiments utilized radiolabeled Dip-AST 11 and found no specific binding in the midgut. Our results suggest that this was likely a result of high endogenous protease activity. In binding experiments with brain or CA membranes, the serine protease inhibitor PMSF and the peptidase inhibitor 1,10-phenanthroline are sufficient to permit the detection of specific binding of ASTs (Yu et al. 1995a). To detect specific binding in the midgut, further inhibition of protease activity was required. We employed Complete™ Mini protease inhibitor cocktail tablets (Boehringer Mannheim) to confer additional protection against proteolytic degradation. These tablets are advertised to inhibit a broad spectrum of serine, cysteine and metalloproteases. Our results support other evidence indicating that 1,10-phenanthroline and PMSF are only marginally effective as inhibitors in the midgut (Bendena et al. 1997; Garside et al. 1997b). It is not surprising that midgut membrane preparations require additional inhibition of proteolysis compared to brain membranes, given that the midgut is a site of synthesis and release of digestive enzymes. It would now be useful to assay brain and/or CA
membranes with the enhanced protection provided by the Complete™ tablets, to determine whether any additional receptor activity could be detected.

*Pseudopeptide analog AST(b)ϕ2*

Our results with the pseudopeptide analog AST(b)ϕ2 were promising, but not conclusive. Although the analog at first appeared to effectively compete with radiolabeled Dip-AST 7, this later proved to be statistically insignificant. There is physiological evidence that AST(b)ϕ2 is able to modulate the secretion and/or activity of digestive enzymes (Fusé et al. 1998). While it is possible that this modulation is not a receptor-mediated event, it is reasonable to assume that the analog is interacting with ASTRs due to its structural similarity to Dip-AST 6. We plan to repeat our competition experiments when we are able to obtain a greater supply of AST(b)ϕ2, and we believe that we will achieve statistical significance with larger sample sizes, particularly if we are able to better control sources of methodological error.

*AST involvement in digestive processes*

The discovery of ASTRs in the midgut adds to a growing body of evidence that points to an important role for ASTs in digestion. The ability of ASTs to inhibit muscular contractions of the hindgut, and to affect the release and/or activities of digestive enzymes in the midgut, may serve a coordinated purpose in enhancing the absorption of nutrients from food. According to the model proposed by Fujita et al. (1981), nutrients entering the midgut from the crop are detected at the apical surface of midgut endocrine cells. Shortly thereafter, ASTs are released from the basal surfaces of these cells (Yu et al. 1995b). Due to their relatively short half-lives, most of the released ASTs probably function in a paracrine manner on nearby cells. The concentration of ASTs in the microenvironment immediately surrounding the endocrine release sites could reach
nanomolar levels or higher, sufficient to activate ASTRs. The ASTRs could be located on neighboring epithelial cells, or on the muscular layers surrounding the gut epithelium. It has been demonstrated that cells in the midgut release increased amounts of the digestive enzymes invertase and α-amylase when incubated with ASTs compared to controls (Fusé et al. 1998). This increase in enzyme production, which would presumably facilitate rapid digestion, may be mediated by ASTRs on epithelial cells. At the same time, ASTs may inhibit the contraction of the hindgut, probably via ASTRs on the surface of muscle cells. This could slow the movement of food through the alimentary canal, permitting enzymatic digestion to proceed for longer periods. An alternate theory has been proposed by Veenstra et al. (1995), in reference to the localization of AST-like immunoreactivity in the posterior midgut of the mosquito Aedes aegypti, just anterior to the pyloric sphincter. They suggest that ASTs might be involved in relaxing the pyloric sphincter following digestion of the blood meal, permitting the movement of the remainder to the hindgut. AST-immunoreactive neurons on the anterior midgut might similarly relax the cardiac sphincter and allow food to move from the crop to the hindgut (Veenstra et al. 1995). In Diploptera, a portion of the anterior midgut is frequently found in a contracted state during dissection, which may suggest that the gut is normally contracted. Inhibition of this contraction by ASTs or other peptides, such as leucomyosuppressin (LMS), may occur only after digestion is completed, allowing a new bolus of food to enter the midgut (Fusé 1998, and pers. comm.).

Evolution of multiple AST functions

Although the Dip-ASTs are best known for, and in fact are named for their ability to inhibit JH biosynthesis by the CA of cockroaches, it is possible that this does not represent their original
or primary function. For example, Dip-ASTs appear to be completely ineffective as inhibitors of JH biosynthesis in Diptera and Lepidoptera. This may represent an evolutionary shift away from Dip-AST regulation of JH biosynthesis in these orders, but it may also indicate that this regulatory mechanism did not develop until after the divergence of these orders from ancestral cockroaches. The "base" function of ASTs may well have been their effects on contraction of the visceral muscles. These effects are seen in cockroaches (Lange et al. 1993, 1995; Duve et al. 1995), and in blowflies (Duve and Thorpe 1994), and immunohistological studies support a role for ASTs in the midgut of mosquitoes (Veenstra et al. 1995). It has also been shown that cockroach ASTs have inhibitory effects on hindgut contraction in the shore crab, Carcinus spp. This may suggest that the myomodulatory function of ASTs preceded the divergence of insects from crustaceans, over 385 million years ago.
Summary

Recent evidence suggests that allatostatins may play a role in the function of the cockroach digestive system. This study was performed (a) to identify receptors for allatostatins in the midgut; (b) to determine whether different members of the allatostatin peptide family have different affinities for midgut receptors; and (c) to determine whether a pseudopeptide analog of allatostatin could compete with native allatostatins for binding to midgut receptors.

1. Mono-iodinated Dip-AST 7 of high specific radioactivity was prepared and partially purified for use in a radioligand-binding assay.

2. The radioligand-binding assay showed that the midgut of the cockroach Diploptera punctata contains a single class of putative receptor for $^{125}$I-Dip-AST 7, with $K_D$ of $14\pm2.2\text{ nM}$ and $B_{max}$ of $1.5\pm0.1\text{ pmol/mg membrane protein}$.

3. Different members of the Dip-allatostatin peptide family have different potencies as competitors for binding to the putative receptor. Dip-AST 7 and 2 are the best competitors, followed by Dip-AST 5, 9, and 11. Dip-AST 13 does not effectively compete for binding.

4. The pseudopeptide Dip-AST analog AST(b)$\phi$2 was not an effective competitor for the putative receptor.
References


