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Mini review

A molecular view of trypsin synthesis in the midgut of *Aedes aegypti*

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Abstract

Ingestion of a blood meal induces two phases of trypsin synthesis in the midgut of *Aedes aegypti* females. The first phase, which encompasses the first 4–6 hours following a blood meal, is characterized by the presence of small amounts of early trypsin. The second phase, which occurs between 8 and 36 hours after blood feeding, is characterized by the presence of large amounts of late trypsin. A specific form of regulation of trypsin synthesis characterizes each phase: early trypsin synthesis is regulated at the translational level, while late trypsin synthesis is regulated at the transcriptional level.

The enzymatic activity of early trypsin plays a unique and critical role in the regulation of late trypsin synthesis. Early trypsin acts like a "sensor". It carries out limited proteolysis of the ingested proteins and, somehow, the products of this limited proteolysis induce synthesis of late trypsin, which is the protease responsible for the majority of the endoproteolytic cleavage of the meal proteins.

Transcription of the early trypsin gene starts a few hours after adult emergence and is under control of juvenile hormone. However, the early trypsin mRNA is stored in the midgut epithelium and remains untranslated until a blood meal is taken. The exact mechanism responsible for initiating translation is presently unknown, but an increase in the size of the amino acid pool in the midgut is sufficient to activate translation of early trypsin mRNA.

The transcription of the late trypsin gene is regulated by uncharacterized proteolysis products generated by the action of early trypsin on the blood meal proteins. Once transcription has been activated, the rate of transcription of the late trypsin gene is proportional to the amount of protein present in the meal. In addition, the amount of late trypsin protein translation is controlled by the amount of amino acid released during digestion. Regulation at both transcriptional and translational levels allows the midgut to adjust the amount of late trypsin with remarkable flexibility in response to a particular meal.

Keywords: Mosquito; Trypsin; Digestion; Regulation; Midgut

1. Introduction

Blood meal digestion by female mosquitoes is a fascinating physiological process. In a few seconds they ingest more than their own weight in blood and then spend the next 36 hours converting the amino acids in the blood proteins into the constituents of their eggs. Proteins are the predominant components of blood and 24 hours after feeding 80% of the ingested protein has been digested (Briegel and Lea, 1975). This is possible because the blood meal induces a large increase in midgut proteolytic activity (Fisk, 1950), with trypsin representing the main endoproteolytic enzyme (Briegel and Lea, 1975). In this mini-review we will summarize our current understanding of the regulation of trypsin synthesis in the midgut of adult *Aedes aegypti* females.

2. The midgut expresses two different trypsins following a blood meal

It is now well established that the midgut of female *Aedes aegypti* synthesizes two trypsin forms following a blood meal. *Early trypsin*, which is produced in small amounts, appears in the midgut within 1 h of the blood

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meal, and disappears by 6–8 h after the blood meal. *Late trypsin*, which is produced in large amounts, begins to appear 8–10 h after the blood meal, and accounts for most of the endoproteolytic activity present in the midgut during blood meal digestion (Fig. 1).

3. Late trypsin

Late trypsin is an adult female midgut-specific protein. It was isolated and characterized by Graf and Briegel (1985, 1989) and Graf et al. (1988, 1991). This is the most abundant endoprotease present in the midgut during blood-meal digestion, reaching, at the peak of protease activity, concentrations of approximately 5–6 μg/midgut (Graf et al., 1988). Immunocytochemical studies localized late trypsin to the midgut and described its secretory pathway (Graf et al., 1986). A monoclonal antibody produced against this protein (Graf et al., 1988) was used to clone the late trypsin cDNA (Barillas-Mury et al., 1991) and the amino-terminal 35 amino acids of the purified protein (Graf et al., 1991) match the sequence of the cDNA (Barillas-Mury et al., 1991). Blood feeding induces expression of the late trypsin gene (Barillas-Mury et al., 1991). Thus, the late trypsin mRNA is not present in unfed females, is first detected 8 h after feeding and reaches a maximum level 24 hours after feeding (Fig. 2). This increase in mRNA levels is followed by an increase in late trypsin protein. All available data indicates the synthesis of late trypsin is regulated at the transcriptional level (Barillas-Mury et al., 1991). Although the gene for late trypsin has been sequenced (Barillas-Mury and Wells, 1993), nothing is known about regulatory elements that control transcription.

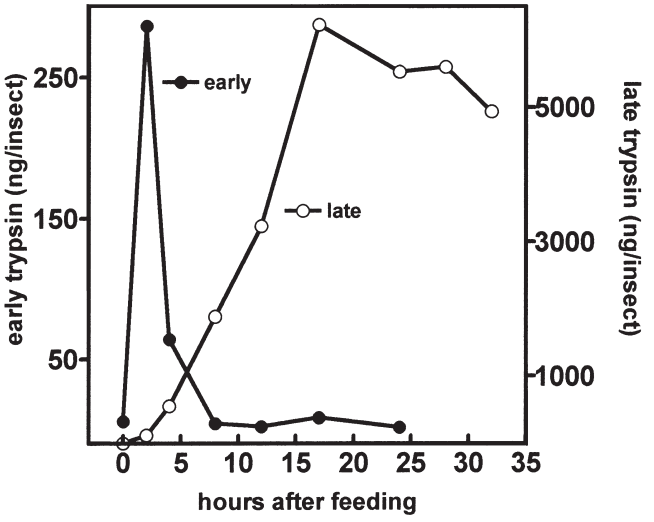

Fig. 1. The mass of early and late trypsin in the adult female *Aedes aegypti* following a blood meal. The data for early trypsin (●) are from Pennington et al. (1995) and the data for late trypsin (○) are from Graf et al. (1988).

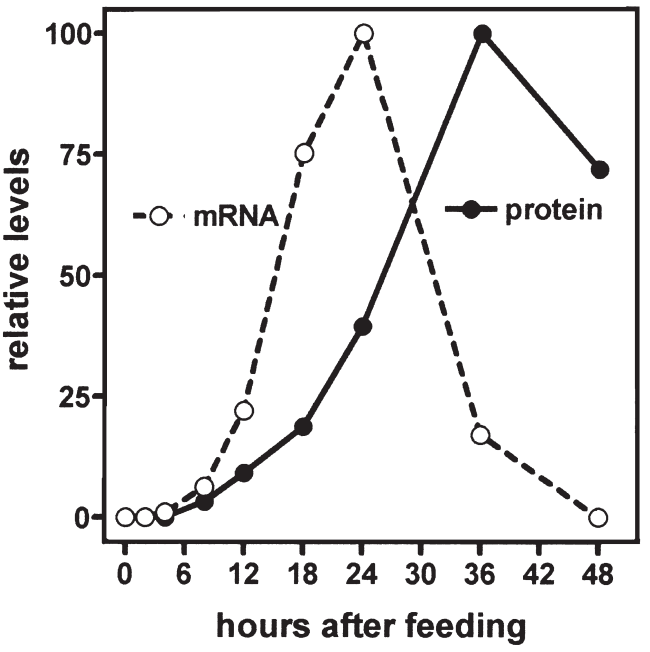

Fig. 2. The relative concentration of late trypsin mRNA (○) and protein (●) following a blood meal. The data are from Barillas-Mury et al. (1991). Note that the time course for late protein appearance is slightly different than shown in Fig. 1, presumably because the data were collected in different laboratories that have different rearing conditions.

4. Early trypsin

Early trypsin is the most abundant midgut protein isolated by benzamidine-sepharose affinity chromatography three hours after blood feeding (Noriega et al., 1996a). The amino-terminal sequence of the early trypsin protein matches that of the 3a1 cDNA for a putative trypsinogen described by Kalhok et al. (1993). The early trypsin mRNA, absent in larvae, pupae and newly emerged females, reaches detectable levels during the first 24 h after adult emergence and attains a maximum level in 2–3 day-old females (Fig. 3) (Noriega et al., 1996b). In spite of the high levels of early trypsin mRNA in the midgut of unfed females, synthesis of early trypsin protein occurs only after ingestion of a blood meal (Noriega et al., 1996b). Translation of the early trypsin mRNA is accompanied by turnover of the early trypsin mRNA and the steady level of the mRNA drops to very low levels within a few hours after feeding (Noriega et al., 1996b). By the end of the gonadotrophic cycle the levels of early trypsin mRNA return to pre-blood meal levels and remain high until the next blood meal is taken. If a second blood meal is taken the entire process is repeated.

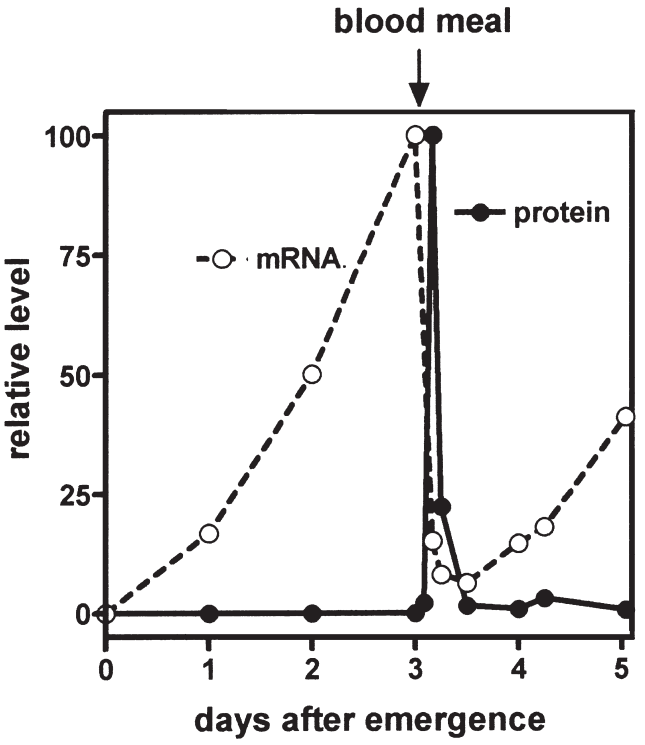

Fig. 3. The relative concentration of early trypsin mRNA (○) following emergence and a blood meal and the relative concentration of early trypsin protein (●) following a blood meal. The mRNA data are from Noriega et al. (1996b) and the protein data are from Pennington et al. (1995).

5. Role of early trypsin in blood meal protein digestion

While it is clear that late trypsin plays a major part in blood-meal protein digestion, the role of early trypsin is less obvious because at maximal levels the enzyme is present at concentrations of only 300 ng/midgut (Pennington et al., 1995). Furthermore, the maximal concentration of early trypsin also occurs at a time when minimal blood meal protein degradation is occurring. (Briegel and Lea, 1975; Felix et al., 1991).

Barillas-Mury et al. (1995) showed that early trypsin activity is an essential part of the signal transduction pathway that activates the transcription of the late trypsin gene. The addition of soybean trypsin inhibitor (STI) to a protein meal prevented transcriptional activation of the late trypsin gene and therefore protein digestion. This inhibitory effect can be overcome by feeding a protein meal that has been partially digested ex vivo with bovine trypsin before adding STI. Thus, the products released during pre-digestion, which mimic the activity of early trypsin in the mosquito midgut, were able to restore transcriptional activation of the late trypsin gene to control levels.

6. Why is such an elaborate regulatory scheme needed?

The synthesis of a large amount of trypsin in the absence of a blood meal could be deleterious to the mosquito. A two-phase digestive response allows the mosquito to assess the quality of the meal via early trypsin before committing to the synthesis of late trypsin. If there is no protein in the meal, or if it has poor digestibility, no free amino acids, peptides or other "activators" will be released and the late trypsin gene will not be transcribed. To our knowledge, this is the first case in which the activity of a digestive protease has been shown to be part of the signal transduction system that regulates the expression of a second gene.

7. Regulation of early trypsin transcription

Transcription of the early trypsin gene is part of the normal post-emergence maturation of the midgut in the adult female and is controlled by juvenile hormone (JH) (Noriega et al., 1997). Abdominal ligation shortly after adult eclosion, which isolates the midgut from the source of JH, the corpora allata, prevents transcription of the early trypsin gene. Low doses of a JH analogue (methoprene) or higher doses of JH III restored the expression of the early trypsin gene in ligated abdomens (Fig. 4).

Several other results are consistent with a role for JH in regulating early trypsin gene expression:

- Methoprene application after feeding caused the amount of early trypsin mRNA in the midgut to reach, within 12 h, levels normally observed in controls at 40–60 h, which is the time when JH levels normally return to pre blood meal levels.
- Topical application of methoprene immediately after emergence eliminates the 10 h lag normally observed before the transcription of the early trypsin gene reached detectable levels.
- Expression of the early trypsin gene can be also induced in pupae by raising them in the presence of methoprene.
- Injecting freshly eclosed adults with juvenile hormone esterase prevents the normal post emergence transcription of the early trypsin gene (Edgar, et al., unpublished).

To our knowledge, early trypsin is the first JH-regulated midgut gene to be described.

Although we have shown that JH regulates the transcription of the early trypsin gene in a stage-dependent, dose-dependent and "head-independent" manner, we do not yet know whether JH directly affects early trypsin gene expression or whether the effect is mediated by

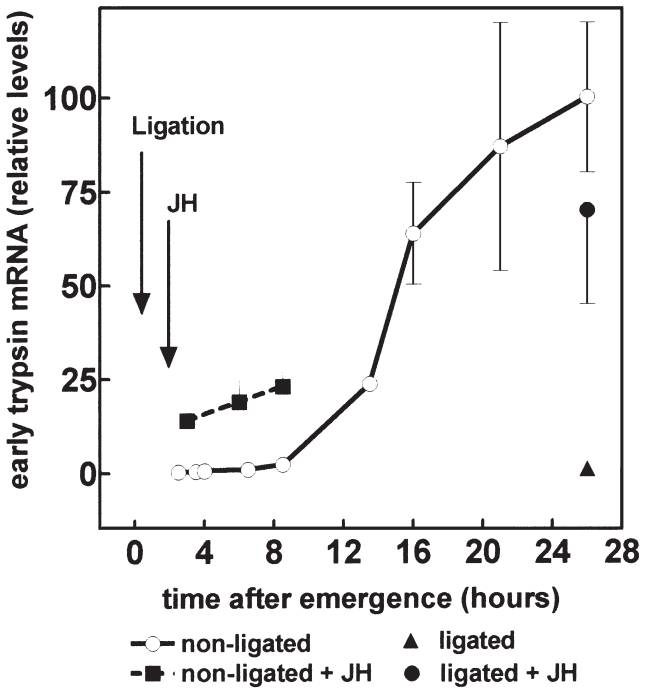

Fig. 4. The effect of juvenile hormone (JH) on early trypsin transcription. The non-ligated insects (○) show the normal time course of early trypsin mRNA appearance in the midgut following emergence. The ligated animals (▲) failed to produce detectable amounts of mRNA. Ligated insects treated with JH (●) produced nearly normal levels of mRNA. Treatment of non-ligated (normal) insects with JH (■) accelerated the appearance of mRNA in the midgut. Taken from Noriega et al. (1997).

some other factor produced in response to JH. The effect of methoprene on the isolated abdomen or in the insect after feeding is blocked by cycloheximide injection suggesting the necessity for protein synthesis before the hormonal effect can occur. Transfusion of hemolymph from methoprene-treated abdomens into freshly ligated abdomens stimulates early trypsin gene expression, showing that the methoprene or an activating factor induced by methoprene is carried via the hemolymph. However, to date, all attempts to reproduce these effects using in vitro midgut cultures have been unsuccessful.

8. Regulation of early trypsin translation

In the unfed midgut there is neither active early trypsin nor early trypsin zymogen. Thus, although the early trypsin mRNA is present, it is not translated. After the first week of adult life, if females do not ingest a blood meal, there is a slow decrease in the steady state level of early trypsin mRNA, but it remains readily detectable for up to a month after emergence (Noriega et al., 1996b).

Feeding "per se" or filling of the midgut is not enough to stimulate early trypsin translation. Although meals containing saline, latex beads or sugar solutions fill the midgut, they do not stimulate early trypsin synthesis. Because these solutions are not isotonic with the mosquito hemolymph, we can conclude that osmotic stress or mechanical distention of the abdomen is not sufficient to induce early trypsin mRNA translation.

Several proteins of variable molecular weight and different amino acid sequences are able to induce early trypsin synthesis; therefore we can exclude the possibility that the presence of a specific peptide derived from a specific blood protein is essential for induction. Feeding amino acid mixtures containing 20, 12, or even 6–8 amino acids induces substantial early trypsin translation. In contrast, single amino acids are essentially incapable of inducing translation of early trypsin. In addition, intra-thoracic injection of an amino acid mixture induced early trypsin translation, while injection of saline or a protein solution had no effect. These data suggest that the size of the free amino acid pool in the midgut somehow regulates early trypsin synthesis. Indeed, when we added ^{35}S-labeled amino acids to a protein meal, the labeled precursors were rapidly absorbed and incorporated into newly synthesized early trypsin.

9. How is the translation of the early trypsin mRNA silenced before feeding?

In vitro translation using mRNA extracted from midguts of unfed and fed females, showed that there are no structural features in the early trypsin mRNA that would impede translation. In addition, a small intron in the gene with an in-frame stop codon was normally spliced from the early trypsin pre-mRNA. There were no recognizable differences in the length of the poly A tail when transcripts isolated from midguts before and after feeding were compared (Noriega et al., unpublished results). It is possible that the translationally inactive state results from the formation of an early trypsin mRNA-protein complex or because the transcript is found in some unusual subcellular localization "away" from the translation machinery. However, at present we favor the hypothesis that early trypsin translation is regulated by the level of amino acid charging of tRNAs. This hypothesis seems attractive considering that an increase in the size of the midgut amino acid pool is sufficient to activate synthesis of early trypsin.

10. Regulation of late trypsin transcription and translation

Several different proteins, e.g., albumin, gamma globulin, when fed individually or in mixtures to mosquitoes are able to induce late trypsin synthesis; whereas meals containing only amino acids, saline or agarose are very poor inducers of transcription. In addition, there are

some proteins that fail to induce late trypsin transcription, e.g., collagen. Thus, transcription of the late trypsin gene is dependent on both the quality and quantity of protein in the meal. Briegel and Lea (1975) showed that the amount of trypsin activity detected in the midgut is proportional to the protein concentration of the meal, but independent of the volume of the meal. The changes in the steady-state levels of late trypsin mRNA during the first 24 h post feeding are also directly proportional to the concentration of protein in the meal (Fig. 5) (Noriega et al., 1994).

The identities of the "activators" released by the action of early trypsin that promote late trypsin transcription are still unknown. The amino acid meals that induce early trypsin translation fail to activate late trypsin transcription to the high levels we normally observe with blood or protein meals (Noriega et al., unpublished results). The experiments with cycloheximide indicate that, besides the activity of early trypsin, some other factor(s) must be translated de novo in order for transcriptional activation of the late trypsin gene to occur (Barillas-Mury et al., 1995).

Some mechanism must also exist which regulates the amount of late trypsin translated in relation to the amount of protein in the meal. When a mixture of radiolabeled amino acids is added to the blood meal, these amino acids are incorporated into newly translated late trypsin (Gooding, 1973). Thus, the amino acids released from protein digestion could be the rate-limiting step for late trypsin translation. This suggestion is supported by the observation that, when the meal was partially digested ex vivo with trypsin before adding STI, the products released during pre-digestion were able to restore transcriptional activation of the late trypsin gene to control levels. However, the presence of STI, which prevents further digestion in vivo, did not allow production of normal amounts of amino acids, and therefore, only very limited translation of late trypsin took place (Barillas-Mury et al., 1995).

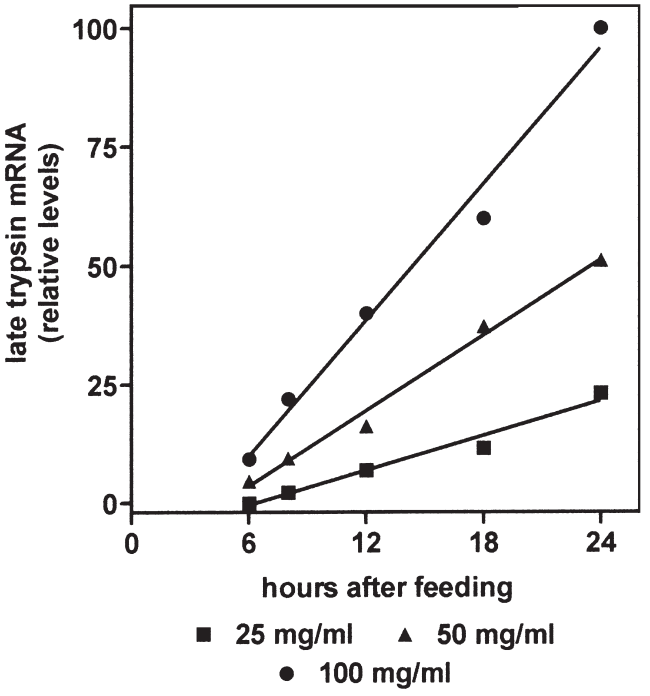

Fig. 5. Proportionality between the rate of accumulation of late trypsin mRNA and the amount of protein present in the meal. The meals contained γ-globulin at concentrations of either 25 (■), 50 (▲) or 100 (●) mg/ml. Taken from Noriega et al. (1994).

11. Mechanisms controlling the synthesis of trypsin in *Aedes aegypti*

The control system(s) that regulate the synthesis and secretion of trypsin may rely on nervous input, endocrine or paracrine activity, or prandial mechanisms (in which elements of the meal interact directly with the secretory cells in the midgut effecting control). The importance of prandial mechanisms has been well established (Briegel and Lea, 1975; Noriega et al., 1994) because the presence of the food in the midgut and the quality and quantity of the meal are essential in the regulation of both phases of trypsin synthesis. The role of paracrine regulators has not been studied, but the presence of more than 500 endocrine cells in the midgut, and the identification of many regulatory peptides that are present in these cells (Veenstra et al., 1995; Veenstra et al., 1997a,b) emphasizes the need for additional research in this area.

- Can the isolated midgut synthesize trypsin in the absence of neural or endocrine signals? Graf and Briegel (1989) reported that early trypsin synthesis was stimulated in a dose-response manner in isolated midguts. We have observed translation of early trypsin in isolated midguts, but the capacity of isolated midguts to transcribe and translate late trypsin is limited (Edgar et al., unpublished).
- What is the role of neural and endocrine mechanisms? We have observed normal late trypsin transcription and translation in decapitated females or isolated abdomens that were given an artificial protein meal by enema (Edgar et al., unpublished). The fact that giving proteins by enema induces trypsin synthesis argues against a regulatory role of foregut receptors.
- In addition, the recognition that trypsin stimulation is dependent on the concentration of the ingested meal and not on the size of the meal suggests that stretch receptors do not play an important regulatory role.
- Downe (1975), Briegel and Lea (1979) and Graf et al. (1998) reported that factors from the brain and the ovary double the amount of trypsin activity.

We can conclude that neural and endocrine signals

have a modulating effect rather than exerting absolute control over trypsin synthesis and secretion.

12. Model for regulation of trypsin synthesis following a blood meal

Based on the work summarized above, we propose the model shown in Fig. 6 for the regulation of trypsin synthesis following a blood meal.

Before feeding the midgut lacks sufficient amino acids to support early trypsin synthesis. Immediately after a blood meal there is an increase in the midgut amino acid pool that induces translation of early trypsin. Where do these amino acids come from? It is possible that other proteases play a role in the initial increase in the amino acid pool following a blood meal, but their importance has yet to be determined. To date chymotrypsin (Jiang et al., 1997), carboxypeptidase (Moskalyk, 1998) and aminopeptidase activity (Graf and Briegel, 1982) have been described.

It is clear that the activity of early trypsin is required in order for transcription of the late trypsin gene to occur. We presume that some digestion product(s) released from the proteins in the meal are the signal for transcriptional activation of the late trypsin gene. We propose that these products activate a regulatory protein (by inducing translation or some other post-translational modification) which in turn activates transcription of the late trypsin gene.

Regulation at both transcriptional and translational levels allows the mosquito to adjust the levels of late trypsin with remarkable flexibility in response to a particular meal. This tight regulation might be particularly

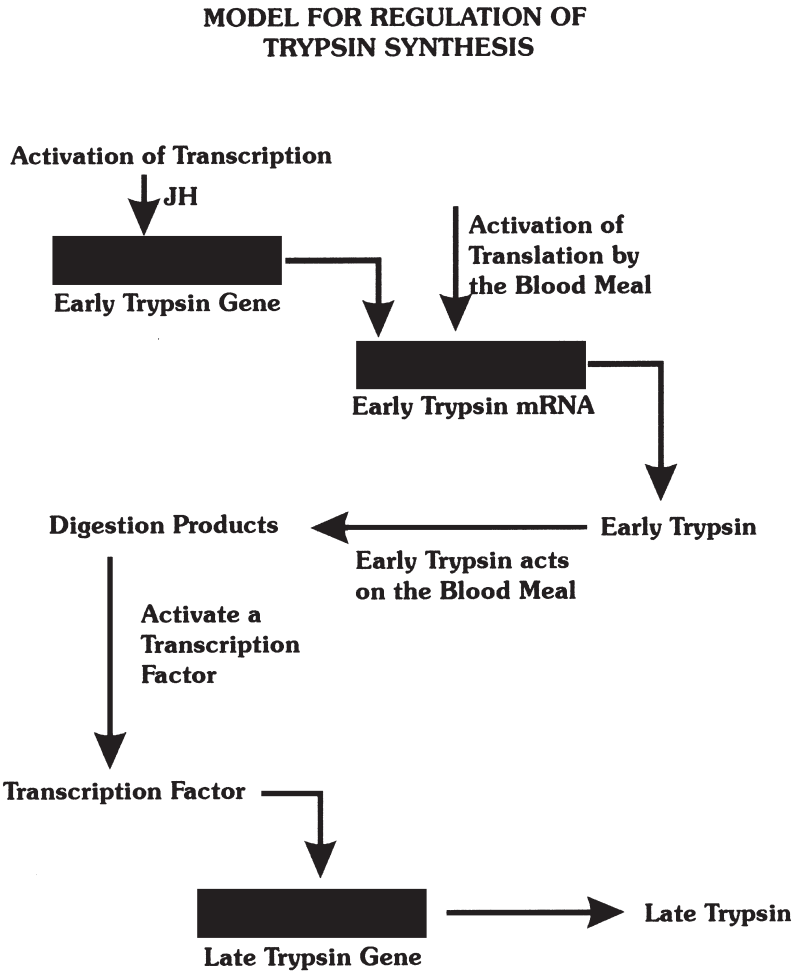

Fig. 6. Working model for regulation of trypsin synthesis in the mosquito midgut. This scheme shows the various stages in regulation of trypsin synthesis in the midgut of *Aedes aegypti*. The *first step* involves transcription of the early trypsin gene under control of juvenile hormone. This is part of the normal post-emergence maturation of the midgut. It is also involved in re-establishing the competence of the midgut to digest a second blood meal following a gonadotrophic cycle. The *second step*, which only occurs following a blood meal, is activation of early trypsin translation. This process requires the presence of free amino acids but the mechanism is unknown at present. In the *third step*, early trypsin carries out a limited proteolysis of the blood meal proteins producing, as yet unidentified, products. These products cause transcriptional activation of the late trypsin gene in the *final step*.

important when most of the meal has already been digested and no additional late trypsin is needed.

13. Preparing for the next blood meal

As blood meal digestion nears completion, the mRNA for late trypsin disappears from the midgut. This seems to be part of a "dedifferentiation" of the midgut, during which the midgut returns to the state existing before the blood meal and remains in this state until the next blood meal is taken. As part of this process, transcription of the early trypsin gene is activated when JH levels rise following egg laying. When a second blood meal is taken the entire process of early and late trypsin synthesis occurs again. The mechanisms underlying this dedifferentiation process are unknown but clearly involves the activity of ribonuclease and proteases, which clear the midgut of the mRNAs and proteins used in digestion and absorption of the first blood meal.

14. Many questions remain!

1. Does JH have a direct effect on early trypsin transcription?
2. What is the molecular mechanism of the "silencing" of early trypsin translation?
3. What is the nature of the "activators" of late trypsin transcription?
4. How is the "proportionality" between protein concentration and trypsin synthesis established?
5. What are the role(s) of other midgut proteases in blood meal protein digestion and/or in regulating trypsin synthesis?
6. What are the relative contributions of prandial and endocrine pathways?
7. What are the regulatory elements in the trypsin genes that are responsible for regulation?
8. What transcription factors are involved in regulating trypsin gene expression?
9. What is involved in dedifferentiation and how is it regulated?

In summary, we hope we have conveyed to the reader the excitement of this unique system. We initiated this project in the hope that analysis of the regulation of trypsin synthesis by blood feeding would contribute to our understanding of the biochemistry and physiology of the mosquito. While this is certainly proving to be the case, the system has many additional features. As a model for understanding the integration of gene regulation and digestive physiology, the mosquito midgut may be unparalleled. This work promises to be seminal to our understanding of the functional significance of the endocrine cells in the insect midgut and the elucidation of the physiological roles of the peptides produced by these cells. Finally, the midgut offers some important opportunities to understand how JH regulates gene expression.

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