Spontaneous Fragmentation of Several Proteins in *Drosophila* Pupae*

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Autoproteolysis is an essential activity in the expression of the entire genomes of a number of viruses. That is, new viruses can be produced only after large polyprotein products translated from the genome or from subgenomic mRNA degrade themselves to the polypeptides necessary for RNA replication or for the construction of new virus particles. We have recently shown that the major heat shock protein of *Drosophila* and a mouse cell line (70 kDa) also undergoes autoproteolysis with the production of specific patterns of smaller polypeptides. We show now that many other proteins in eucaryotic tissues also have a potential for self-degradation. We suggest that special coding regions in many genes may have important roles in both protein turnover and in the production of regulatory peptides.

Proteases occupy a unique niche among the enzymes in that they act upon molecules of their own kind, including themselves. Such autoproteolysis has been known for a very long time as in the case of pepsin (1). This well known digestive enzyme can be produced by autoproteolysis of the precursor pepsinogen to yield pepsin and, at the same time, a peptide that serves as a pepsin inhibitor. Since these early observations, there have been numerous and often controversial reports of autoproteolysis, but it is difficult to distinguish between self-destruction and destruction due to small amounts of contaminating enzymes in most protein preparations. However, there are now well established examples of autoproteolysis derived from studies of some of the RNA viruses (2-7). In some of these cases, the entire genome of the virus is translated in a single piece. In other cases, polypeptides are translated from genomic or subgenomic RNAs that represent less than the entire genome, but they may be quite large nevertheless. Protein components to be used for specific purposes in virus construction or in RNA replication are then cut out of the initial polyprotein by action upon itself. Indeed, evidence has been presented to show that one such polyprotein has two active sites for autoproteolysis (8). Also recently, evidence has been presented in favor of autoproteolysis as an important property of the lexA protein and the phage λ repressors in *Escherichia coli* (9). In higher organisms, reasonable evidence still exists for autoproteolysis as a significant activity in the cascade of reactions that leads to complement.

Experimental Procedures

Biological Material—*Drosophila* pupae were obtained from mass cultures (13) of an Oregon-R strain, and they were staged for synthesis of proteins (15, 16). Actin from a mouse cell line was obtained as described elsewhere (12).

Sample Preparations—Dissected whole wings (17) from pupae were incubated for 30 min in moist chambers with labeled amino acids dissolved in MOPS buffer (14). [3S]Methionine (specific activity, 1000) was used at a level of 5 μCi/ml in 5-20-μl aliquots. [3H]Leucine and [3H]lysine were used at the same level, although specific activities were only about 120 μCi/mM. All three labeled amino acids were obtained from New England Nuclear.

After labeling, wing samples were transferred to Eppendorf tubes (0.4 ml) and heated with vortexing with 50 μl of SDS sample buffer for 3 min. Debris was removed by centrifugation, and clear supernatant solutions were applied directly to gels for electrophoresis.

For in vitro digestions, labeled wing samples were ground with buffer in glass grinders in ice. After centrifugation, 10-μl aliquots (equivalent to three wing pairs) were removed and diluted with 50 μl of SDS sample buffer. These were heated in boiling water for 3 min and then used directly as samples for electrophoresis.

Samples for protein digestion in intact tissue were prepared by labeling whole wings for 30 min followed by washing five times with MOPS buffer. The wings were then incubated further in MOPS buffer, and aliquots of six wing blades were removed at different time intervals for solution in sample buffer and preparation for electrophoresis.

The abbreviations used are: SDS, sodium dodecyl sulfite; MOPS, 3-(N-morpholino)propanesulfonic acid; BSA, bovine serum albumin; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane; DTT, dithiothreitol; TEMED, N,N,N',N'-tetramethylethylenediamine.
FIG. 1. Unstable proteins in differentiating wing tissue. The sample for each of the nine panels in the figure was prepared from three wing pairs dissected from pupae of the stage shown (in hours after pupariation). Each sample was labeled for 30 min in 5 μl of MOPS buffer (14) containing 25 μCi of [35S]methionine (specific activity, 1000). Samples were then extracted in 50 μl of SDS sample buffer, centrifuged, and run for 16 h on a gradient acrylamide gel as described earlier (14). Strips containing the labeled proteins in each lane were then cut from the wet gel and equilibrated for 1 h at 25°C with shaking in equilibration buffer (stacking gel buffer diluted 1 → 4 and containing 2% SDS, 0.01 M DTT, and bromphenol blue to a strong color). The strips were rinsed quickly with distilled water and placed at right angles 1-1.5 cm from the top of the glass plates to be used to form the second gradient gel. During the additional incubation period of 7 h, the gradient gel was pumped and the strip was embedded in stacking gel containing 1% SDS. Electrophoresis was carried out for 16 h at constant current (16 mA/220 sq mm of surface area). Gels were then trimmed, partially dehydrated by shaking in destaining solution, and dried in vacuo at 70°C. In each panel, the upper diagonal line shows an exposure to x-ray film in hours, and the lower diagonal shows an exposure of the same gel for the same number of days. Thus, degradation products of specific labeled proteins show in vertical rows, and amounts of products can be compared directly with origin components in the upper diagonal on a 1:24 basis. Mapr proteins that show degradation are marked on the upper diagonal in approximate kilodaltons.

RESULTS

Unstable Proteins in Differentiating Wing Tissue—We have described in detail elsewhere (14-16) the extensive changes that occur in the patterns of protein synthesis during the synchronous differentiation of wing cells in Drosophila pupae. We now make use of the same system to survey the potential for self-degradation of the many proteins that were observed. A summary of the results is presented in Fig. 1. Each of the
nine panels of the figure was derived from labeled wing tissue of the developmental stage shown as hours after puparium formation. Under the conditions of this experiment, 13 different proteins yield vertical rows of degradation components. These are marked above the origin spots on the upper diagonal in numbers representing approximate masses in kilodaltons. As shown, the sizes of the more unstable proteins vary from 20 to more than 130 kDa. The component marked 44 in the panels at 47, 81, and 88 h is the well known protein actin which is produced most actively in wings around 45 h and again in the 80-h range (19). None of the other abundant but unstable proteins in the wings has yet been identified as to function.

Autoproteolysis—Although it is likely that each of the 13 proteins which shows degradation in the two-dimensional gel system used for obtaining the data in Fig. 1 undergoes autoproteolysis, additional evidence is desirable. To this end, we have examined further the properties of six abundant components that are synthesized in Drosophila wings during the 80–85-h period. These are marked with approximate molecular masses (in kilodaltons) on the one-dimensional gel shown at the left in Fig. 2. Four of these were rerun in one dimension, incubated for 8 h, and then run in the second dimension. As shown, two of the components undergo degradation (91 and 56 kDa), while the other two (160 and 34 kDa) are relatively inert. To explain these results in terms of contaminating proteases, such proteases would have to have been present in the 1-mm bands cut from the 91 and 56 positions and not in the 160 and 34 positions. Furthermore they would have had to remain with the 91- and 56-kDa components through the first rerun of each original band.

Two of the six components considered in Fig. 2 were examined further with the results shown in Figs. 3 and 4. To obtain the data shown (Figs. 3 and 4), duplicate samples were run on a gel to give the patterns of strongly labeled products}

![Fig. 2. Reruns of unstable proteins. Samples of wing extracts (from 85-h pupae) for electrophoresis were prepared as described in Fig. 1. An autoradiogram from the first dimension is shown at the left in the figure. Major proteins to be discussed further in this article are indicated by approximate molecular masses (in kilodaltons). For the first rerun, bands 1-1.5 mm wide were cut from a wet gel strip of the first dimension. These small gel blocks were equilibrated and incorporated into a second gradient gel as described for the strips in Fig. 1. After running this gel, a strip was cut from the position of each block, and the process was repeated for each strip to show the second reruns. Autoradiograms for four of the proteins under consideration are shown at the right in the figure. The other two, the 130- and 44-kDa proteins, are considered in more detail in Figs. 3 and 4.](image)

such as that shown at the left in Fig. 2. Narrow bands corresponding to the 130- and 44-kDa components were cut from the wet gel. These small blocks (1 × 10 mm) were incubated in the equilibrium buffer (8 h), incorporated into a new gel, and rerun, again in duplicate. Strips of wet gel corresponding to each block were then cut out. One of the duplicate strips from each component was then incubated in equilibrium buffer (8 h) and incorporated at right angles at the top of a third gel. These were run and dried to give the autoradiograms shown under part c in Figs. 3 and 4. The
other duplicate strips were each cut in two pieces. The portions containing the degradation products of the 130- or the 44-kDa proteins were dried and exposed to yield the patterns shown in Figs. 3 and 4, column b. The pieces containing the remaining unchanged original components were used as samples for focusing gels, and these were carried through the standard two-dimensional system (19). The portions of these final gels which show the focused products are shown in column a, Figs. 3 and 4.

These results provide several additional facts that support further the concept of autoproteolysis. In Fig. 3, column c, the large degradation product (s) also undergoes degradation to produce a pattern similar to that of the original 130-kDa protein. Furthermore, as shown in column a, each of the several focusing isoforms of this component gives rise to the prominent s product. In addition, with longer exposures of the focused gel we have observed that the less abundant products (as seen in column b and part c of Fig. 3) also follow the isoform pattern. Thus, if the degradations observed were due to a contaminating protease, the contaminant would have to move with s in part c and as each of the isoforms in column a. Autoproteolysis provides a reasonable explanation of the results.

The sixth component (Fig. 2) subjected to additional scrutiny is the well known protein actin, and the data are summarized in Fig. 4. Again as in Fig. 3, the degradation pattern of a first rerun is shown in column b and in the diagonal in part c. The same typical pattern (self-fingerprint) from the second rerun is shown in the vertical in part c. These patterns (column b and part c) are essentially identical, but that in column a is only similar. The reason is that this particular actin sample was derived from a mouse cell line rather than Drosophila tissue. It was carried through a rerun in one dimension before focusing. Thus, despite the fact that cell lines in general show strong general protease activities, as opposed to wing tissue from Drosophila, the initial extraction prepared directly with the result shown in part c. For part c, 20 pairs of wings (85 h) were labeled with [35S]methionine and extracted in 80 μl of bis-Tris-HCl buffer, pH 7.5 (0.05 M), containing 0.1% Triton X-100. The supernatant solution from 5-min centrifugation at 10,000 rpm was incubated at 25 °C. Aliquots (10 μl) were taken at intervals as shown at the right, diluted with 50 μl of SDS sample buffer, and run on the standard gradient gel. All four samples (0, 1.5, 4, and 8 h) were run on the same gel at the same time. The cut sections shown are from autoradiograms of the same gel exposed for different time periods. This was done because the darker bands did not show the degradations clearly by direct inspection if equal time exposures were used.

Degradation in Vitro—At the present time, we have no satisfactory criteria for distinguishing autoproteolysis from general protease action in a non-denatured system. However, we have observed repeatedly that wing and some other tissues that are in the process of differentiation in pupal stages do not exhibit unlimited protease activity. Thus, we have again made use of this system to compare stabilities of specific protein components in SDS and in buffer extracts. Results are summarized in Fig. 5. Strip a shows a one-dimensional gel from 85-h wings, and panel b shows the result of incubation of an identical strip (7 h) in SDS before running the second dimension, also in SDS. The four strips at the bottom (part c) show the disappearance of wing proteins on incubation in a non-denaturing buffer. It is clear from these results that the same set of proteins are affected in this system as in the denatured system. The 160-kDa component that is relatively stable (Fig. 2) also degrades, but the 34-kDa polypeptide does not. The 70-kDa protein is also included here. This is the 70-kDa heat shock protein (which is produced in 80-h wings without heat shock), and its stability has been considered elsewhere (12). It is of interest to note also an accumulation of degradation products just to the right of actin (44 kDa). These appear to be products from actin, and indeed the larger one may be the 39-kDa component described several years ago (20).

pH and Inhibition—Protein degradation in gels in the presence of SDS occurs at pH 6.8 (the equilibration buffer) and at pH 8.8 (the running buffer), but we have made no attempt to evaluate pH and inhibitor effects in these circumstances, because specific concentrations depend on band width and spot size and shape. On the other hand, reasonable kinetics can be carried out in the non-denaturing system, as shown in Fig. 5, part c. In that example, as in the series in Fig. 6, none of the proteins affected disappears completely. All are retained to the extent of 20–30%, even on incubations as long as 24 h. We presume that this is due to product inhibition, but we have not yet evaluated this probability. It is worthy of note that the serine protease inhibitor aprotinin (Sigma) has no effect on any of the protein losses shown in Figs. 5 and 6 at a level of 20 trypsin units/ml. With respect to pH effects, it is clear simply by inspection of the data in Fig. 6 that each protein that shows instability does so over the whole range examined, but each has its own characteristics. The 130-kDa protein shows the most striking solubility difference with good extraction at high pH, but it seems to degrade over the whole range. The 56-kDa component seems to degrade most rapidly at pH 6.6, whereas the 70-kDa component goes most rapidly at pH 8.6. The 34- and 30-kDa proteins are stable over the whole range, although both are less soluble at pH 8.6. These specific individual details can be quantitated, but the important conclusion in the present context is that non-specific and general protease activity is not observed in these
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extracts of wing tissue over the pH range of 6.6-8.8. An additional series at pH 5.6 was included in this original experiment, but the data are not shown because many major proteins of interest are not extracted at this pH. No general proteolysis was evident at pH 5.6, in any case.

Intact Tissue—Developing wings from Drosophila pupae label quickly and continue normal differentiation for several hours in a synthetic medium and thus lend themselves well to pulse-chase experiments. Results from such an experiment using wings from 80-h pupae are shown in Fig. 6. A aliquots (10 μl) were diluted with 50 μl of SDS sample buffer and heated 3 min in a boiling water bath. Approximate molecular masses (in kilodaltons) of several of the abundant proteins are given at the left. The pH of the buffer for each set is given at the top and the time intervals for incubation (in hours) is given at the bottom.

with heat shock proteins from Drosophila, mouse, and hamster cell lines (12), but in these cases also a number of other proteins behave in a similar manner. We have no information on the specific role of SDS in the protein degradations observed, but it is of course obvious that this substance causes extensive denaturation on the one hand, but it must also fix polypeptides in some relative stable conformations on the other. We observed many years ago such a modification of cytochrome c by addition of palmitic acid, and this occurred in vivo as well as in vitro (21). In any case, autoproteolysis only requires conformational changes sufficient to overcome the activation energy to initiate hydrolysis of peptide bonds. As described in elegant fashion by Karplus and McCammon (22), proteins are dynamic molecules with constantly changing conformation. It would need only rare combinations of changes to activate significant amounts of autoproteolysis.

It seems from these data that the stability of a number of proteins in vivo does depend on complexing to a considerable extent. Actin, for example, is unstable in vitro and in SDS despite the capacity for synthesis of all the more stable proteins as well as several new ones, as would be expected from studies on intact, whole animals (14).

It is clear from the data presented that several of the proteins that are unstable in the presence of SDS are also unstable in dilute nondenaturing systems. Because these degradations in the latter case do not go to completion and general proteolysis is not evident in differentiating wing tissue from Drosophila pupae, we consider it likely that autoproteolysis is the degradative process here too. The lack of effect by the serine protease inhibitor aprotinin is in keeping with this probability, because the major proteases of Drosophila have the properties of trypsin (23). The data in this report concerned with the possibility of autoproteolysis as a general process in vivo suggest that it may be a realistic concept. Selective loss of specific proteins does occur. This could

FIG. 6. Protein stability in vitro at different pH values. Samples were prepared from 10 wing pairs, each (85 h) labeled with [35S]methionine in small glass grinders with 80 μl of buffer (0.05 M TRIS, bis-Tris, or mixtures thereof, 1% Triton X-100 adjusted to pH values with HCl). Supernatant solutions from 5 min centrifugation at 10,000 rpm were incubated at 25 °C. Aliquots (10 μl) were diluted with 50 μl of SDS sample buffer and heated 3 min in a boiling water bath. Approximate molecular masses (in kilodaltons) of several of the abundant proteins are given at the left. The pH of the buffer for each set is given at the top and the time intervals for incubation (in hours) is given at the bottom.

FIG. 7. Protein stability in vivo. Whole wings from 80-h pupae were labeled for 30 min with [3H]leucine and [3H]lysine (25 mCi/ml; specific activity, approximately 120 Ci/mM) in MOPS buffer. They were then washed 5 times with buffer and incubated at 25 °C. Each sample contained three wing pairs. The control sample was incubated 7 h in buffer and then labeled for 30 min. All samples were extracted with hot SDS sample buffer. Experiments not shown demonstrated that essentially all of the label in the wings is incorporated into protein within the first hour. Approximate molecular masses (in kilodaltons) for major proteins are given at the left and incubation times (in hours) for each sample are shown at the bottom. The control is shown at the right (7-C).
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Prior to our earlier publication (12) concerned with the instability of the 70-kDa heat shock protein, we considered extensively possible influences of various additives commonly used with acrylamide gels. Included were SDS, urea, DTT, TEMED, ammonium persulfate, acrylamide, glycine, and Tris. EDTA and the protease inhibitor aprotinin were also tested. None of these components had additional effects on the protein fragmentation described. Some of these substances were tested in combination, but we had not considered directly the catalytic effects of heavy metal ions as reported by Kim et al. (29). This has now been done and representative data are presented in Fig. 8. In the upper panel of the figure, some degradation of BSA is shown to occur with no additives (about 1% as estimated from the intensities of silver stain). No more and apparently less degradation occurs with iron plus DTT, or with DTT alone. BSA is in fact one of the "stable" proteins as reported by Kim et al. (29).

As shown in the two lower panels in Fig. 8, neither EDTA nor iron plus DTT has much if any effect on the fragmentation of the several unstable proteins in wing extracts of Drosophila pupae (see Fig. 5 for comparison).

We conclude from these experiments that the protein degradations that we observe in the acrylamide gel matrix and in the presence of SDS are not due to catalytic effects of trace heavy metals. We note also that fragmentation of BSA occurs under our conditions in the absence of any radioactive label, and we have observed this previously with several other proteins. Thus, although some degradation due to tritium decay in the labeled samples remains a possibility, it is clearly not a primary cause of the protein instability observed.

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APPENDIX

Fig. 8. Trace metals. To assess the potential effects of heavy metals on the observed protein degradations, gel strips from a first dimension were equilibrated for the second dimension with various additives and combinations thereof. Concentrations were: DTT, 15 mM; EDTA, 1.5 mM, and FeCl₃, 30 μM. BSA was used at 1 mg/ml with 50-μl samples. The top panel in the picture shows results obtained with BSA and silver stain. No radioactivity was present. The single column at the right shows a first dimension gel strip and the panel at the left shows second dimension results with the additives indicated. Results in the lower panel were obtained with wing extracts as described for Fig. 5, except that the equilibration buffer for the second dimension contained the additives indicated. The upper diagonals show the autoradiograms obtained in a given number of hours and the lower diagonal and vertical patterns show results from exposure of the same gels for the same number of days (24 times longer).

happen through the ubiquitin system (24, 25) in the intact tissue (Fig. 7), but it is extremely unlikely in the in vitro systems (Figs. 5 and 6). Furthermore, it would be most convenient if the potential for autoproteolysis were built into the coding region of each gene that gives rise to an unstable protein product. Perhaps stability can be achieved in such a case by formation of polymers or other complex formations. Because any proteolysis probably involves tertiary configurations, it is not very likely that the potential for such instability can be recognized as a simple base sequence within a coding region. However, the configuration around the serine of serine proteases has already been used in this fashion (26, 27).

With regard to the possible biological functions of autoproteolysis, one is clear in the construction of certain viruses (2–7). This is a regulatory function, and similar but less obvious situations may exist in both procaryotes and eucaryotes. Autoproteolysis is surely a prime candidate for regulation of turnover. Furthermore, the products of turnover may well have special functions such as those of peptide hormones and/ or neurotransmitters. It would be of considerable interest to examine some of the polyproteins (28) for autoproteolytic potential and to look for biological functions for peptides produced from turnover in general.
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