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Self-degradation of heat shock proteins

(protease/stress proteins/*Drosophila*/mammalian cells)

HERSCHEL K. MITCHELL*, NANCY S. PETERSEN†, AND CAROLYN H. BUZIN‡

*Division of Biology, California Institute of Technology, Pasadena, CA 91125; †Department of Biochemistry, University of Wyoming, Laramie, WY 82071; and ‡Division of Cytogenetics and Cytology, City of Hope Medical Center, Duarte, CA 91010

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ABSTRACT **The 70-kDa heat shock protein of *Drosophila* decays *in vivo* at a much faster rate than other abundantly labeled proteins. Degradation also occurs *in vitro*, even during electrophoresis. It appears that this degradation is not mediated by a general protease and that the 70-kDa heat shock protein has a slow proteolytic action upon itself. Heat-induced proteins in CHO cells and a mouse cell line also degrade spontaneously *in vitro*, as do certain non-heat shock proteins from *Drosophila* tissues as well as the cell lines.**

Since the discovery of the heat shock proteins (hsps) in *Drosophila* (1), it has been demonstrated that all of a great variety of organisms and living cells respond to heat shock in a fashion similar to that of *Drosophila* (2). That is, a brief treatment at an elevated temperature induces a small number of specific genes to produce mRNAs and, in turn, a similar number of proteins are synthesized in abundance. Furthermore, synthesis of some or all of these proteins is induced by many other forms of stress (2), both physical and chemical, and it appears that one or more of the proteins may have a physiological role in dealing with resistance to stressful situations. (For reviews, see refs. 2–5.)

Much attention has been given to the problem of gene organization and amino acid sequences of the major hsps of *Drosophila* as derived from DNA sequences (6, 7), but details of properties and functions of the proteins are still unknown. One facet of this question of function concerns the disposition of the hsps once they are formed in response to a stress. The 70-kDa hsp of *Drosophila* disappears in larvae over a period of about 12 hr (8). Instability of 70-kDa hsp *in vitro* also has been reported (9–11). As we describe in this report, this protein evidently has a limited proteolytic action upon itself. We show also that Chinese hamster ovary (CHO) cells and a mouse cell line each produce at least one 70-kDa component that behaves similarly to the 70-kDa component of *Drosophila*.

MATERIALS AND METHODS

Drosophila larvae and pupae were from mass cultures (12) of an Oregon R stock. Third-instar larvae (100 hr from egg-laying) were injected with radioactive amino acids as described (13) and then kept in a food vial until use. Wing tissue from 70-hr pupae was used for most of the experiments because it is quite homogeneous with respect to cell types and contains cells that are differentiating but not dividing (14–16). In most experiments we used [^{35}S]methionine (specific activity about 1000 Ci/mmol; 1 Ci = 37 GBq) at a concentration of 1 mCi/100 μl in 3-(*N*-morpholino)propanesulfonate (Mops) buffer (14). Some experiments were done with [^{3}H]leucine at the same radioactivity level.

CHO and mouse L-929 cells were grown in monolayer culture in RPMI-1640 medium containing 10% fetal calf serum. For heat shock, 35-mm plastic tissue culture dishes each containing 1–2 × 10^6 cells were heated at 45°C for 20 min. Cells were shifted to 37°C for 3 hr, then incubated for 1 hr at 37°C with [^{35}S]methionine (200 μCi/ml, ≈1000 Ci/mmol) in Eagle's balanced salt solution. Cells were washed twice with phosphate-buffered saline and solubilized on the plate with 100 μl of O'Farrell's lysis buffer (17). Aliquots were diluted in hot $NaDodSO_4$ sample buffer (1% $NaDodSO_4$/10 mM dithiothreitol/0.125 M Tris Cl, pH 6.8/10% glycerol/bromophenol blue) as described in *Results* for *Drosophila* wing tissue. Electrophoresis was carried out in two dimensions (both $NaDodSO_4$/PAGE).

Unless otherwise stated, we used gradient gels as described (14). Proteins were visualized by fluorography. For reruns of gel blocks or gel strips, the sample gels were equilibrated for 30 min in stacking gel buffer diluted 1:4 and containing 2% $NaDodSO_4$ and 1 mM dithiothreitol. They were then embedded in standard stocking gel containing 1% $NaDodSO_4$. It is convenient to sandwich the gel samples between the glass plates before the separation gel is formed. In the one case (Fig. 2) involving isoelectric focusing, the first dimension separation was carried out essentially as described (18). In all of these experiments, protein components were not extracted from the gels at any time. This virtually eliminates the possibility of contamination of samples with proteases from external sources.

RESULTS

Degradation *in Vivo*. The 70-kDa hsp is induced within the first half hour after heat shock (35°C for 30 min) of *Drosophila* larvae and then disappears to a low level by 12 hr (Fig. 1). The radioactivity in other hsps (68-kDa and those in the 20-kDa range) remains essentially constant over this time period as does that in most of the non-hsps. The 84-kDa hsp is also synthesized at 25°C, and we have not evaluated its stability in this system. These results show that 70-kDa hsp is disposed of *in vivo* by some mechanism that apparently does not affect most other proteins. It is thus necessary to postulate either that the larval tissues have a component that acts specifically to remove the 70-kDa protein or that this hsp is responsible for its own destruction. As demonstrated below, self-destruction occurs *in vitro* and may explain degradation *in vivo*.

Stability in Gels. In contrast to extracts of *Drosophila* cell lines or larval salivary glands (unpublished data), those from differentiating wing tissue (pupal stages) are remarkably free

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Abbreviations: hsp, heat shock protein; Mops, 3-(*N*-morpholino)-propanesulfonic acid.

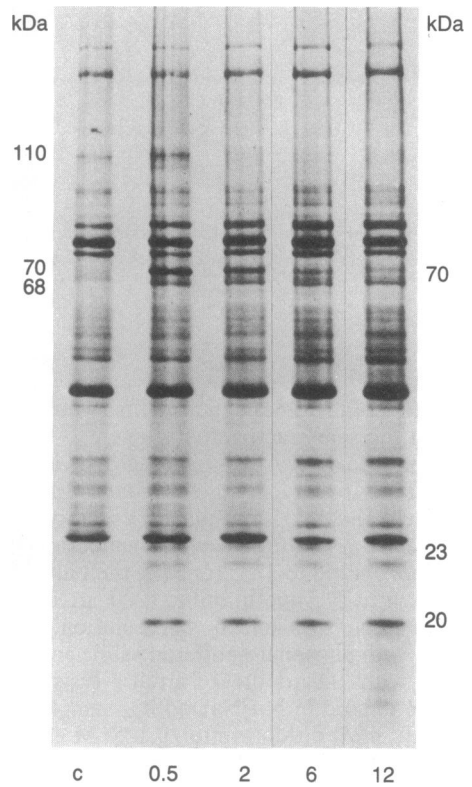

FIG. 1. Stability of the 70-kDa hsp *in vivo*. Larvae (84 hr) were heat-shocked at 36°C for 30 min, immediately injected with [^{35}S]methionine (1 μCi/0.1 μl), and then placed in a food vial at 25°C. Samples (three larvae) were taken 0.5, 2, 6, and 12 hr after injection, ground in sample buffer, heated at 100°C for 5 min, and centrifuged and aliquots (50 μl) were electrophoresed. Lane c: sample from larvae that were not heat-shocked.

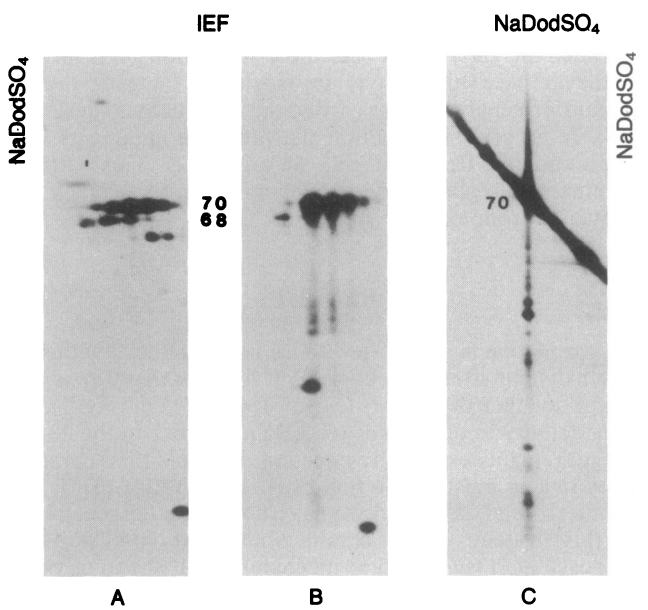

FIG. 2. Two-dimensional electrophoresis of proteins in the region of the 70-kDa hsp. (*A* and *B*) Isoelectric focusing (IEF, acidic end to the right) in combination with NaDodSO$_4$/PAGE. Proteins were from pupal wing tissue labeled with [^{35}S]methionine and [^{3}H]leucine. (*C*) NaDodSO$_4$/PAGE in both directions. Only the gel regions showing the 70-kDa hsp are presented. Numbers show positions of hsps. Vertical rows of products below 70-kDa show degradation components. Sample in *A* was incubated first without denaturation and then boiled with NaDodSO$_4$ sample buffer for 4 min. After focusing, it was equilibrated only 0.5 hr before NaDodSO$_4$/PAGE. Sample in *B* was denatured in NaDodSO$_4$ just after labeling and equilibrated for 3.5 hr after focusing.

of protease activity. Wing tissue from 51-hr pupae was heat-shocked at 37°C for 30 min and then incubated for 30 min at 25°C with [^{35}S]methionine and [^{3}H]leucine. After labeling in Mops buffer, aliquots of the extract were incubated in Mops buffer without radiolabel for up to 1.5 hr and then heated for 4 min in NaDodSO$_4$ sample buffer. They were then immediately prepared for isoelectric focusing in 8 M urea. After NaDodSO$_4$/PAGE in the second dimension, relative amounts of radioactivity in hsps and other proteins were identical regardless of incubation time prior to denaturation. It was clear from these results that extracts of the wing tissue have very little general protease activity. Only the 1.5-hr sample is shown (Fig. 2*A*).

A contrasting result which shows degradation of at least three of the 70-kDa hsp isoforms is shown in Fig. 2*B*. This sample was dissolved directly in hot NaDodSO$_4$ sample buffer immediately after labeling and then diluted in 8 M urea

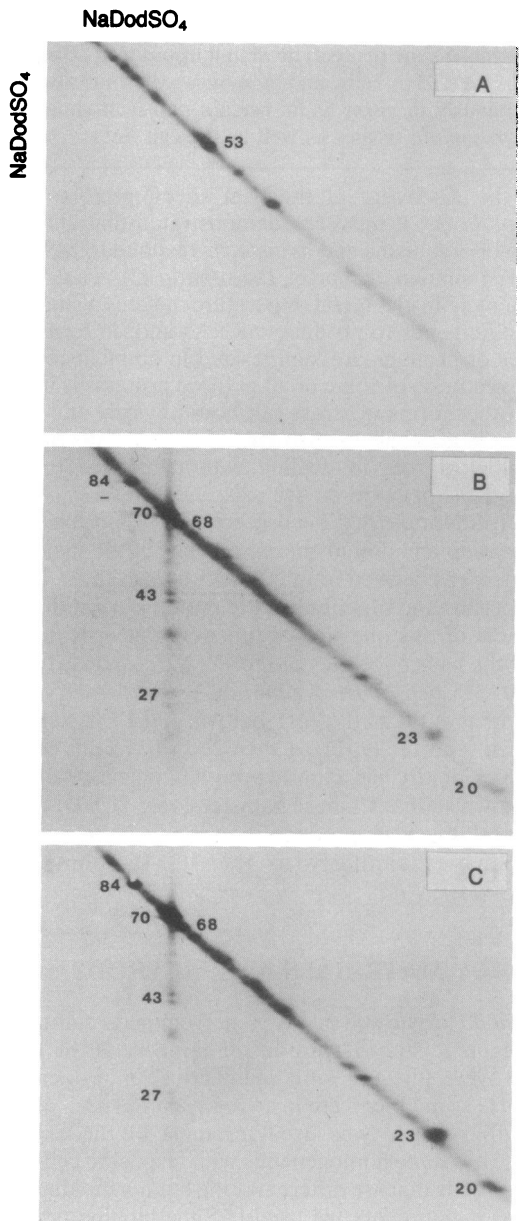

FIG. 3. Two-dimensional NaDodSO$_4$ gels. The samples were from wings of 72-hr pupae. Wing tissue was incubated with a mixture of [^{35}S]methionine and [^{3}H]leucine for 30 min at 25°C either without prior heat shock (*A*), immediately after a 30-min heat shock at 34°C (*B*), or 6 hr after a 30-min heat shock at 40.7°C. Numbers give sizes in kDa.

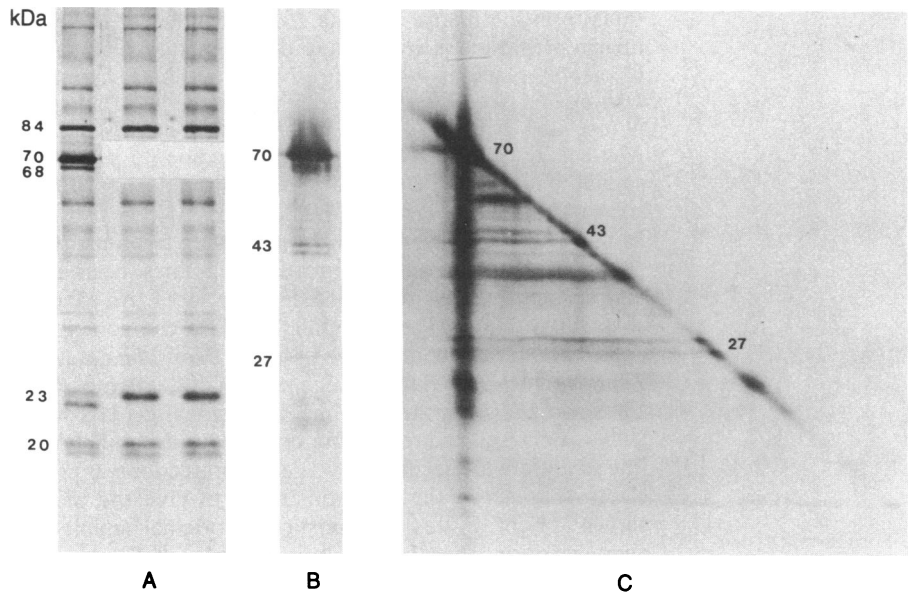

FIG. 4. (*A*) NaDodSO$_4$/PAGE of replicate samples from heat-shocked (37°C, 30 min) 72-hr pupal wings. As shown, the initial blocks excised included both 68- and 70-kDa hsps. (*B*) Direct reelectrophoresis from one of these blocks that had been incorporated in a new gel after an incubation at 25°C for 6 hr. (*C*) Two-dimensional NaDodSO$_4$/PAGE of a block as in *B* except that the 68-kDa hsp was trimmed away before the rerun. Both the gel block and the gel strip (from the second electrophoresis; i.e., from the first dimension in *C*) were equilibrated for 30 min in NaDodSO$_4$ sample buffer (pH 6.8) before incubation for 6 hr. The autoradiogram in *C* was exposed five times as long as that in *B*.

for isoelectric focusing. After focusing, the tube gel was equilibrated for 3.5 hr in neutral NaDodSO$_4$ buffer (sample buffer with 2% NaDodSO$_4$) before the second dimension was run. From a comparison of Fig. 2 *A* and *B*, it is clear that the degradation of the 70-kDa hsps is far more extensive with the longer incubation in NaDodSO$_4$ between the focusing and the gradient gel steps. The activity that digests the 70-kDa protein focuses with the 70-kDa protein in the isoelectric focusing gel.

We will also show that extensive degradation occurred during electrophoresis of denatured samples. The results in Fig. 2*A* along with controls not shown gave evidence that various gel or sample buffer components (such as persulfate, *N,N,N′,N′*-tetramethylethylenediamine, or dithiothreitol) are not involved in the decay of the 70-kDa hsp.

The picture in Fig. 2*C* is derived from a two-dimensional gel in which NaDodSO$_4$/polyacrylamide gels with identical gradients were used in both dimensions. A lane from the first gel was cut out and laid across the top of the second. In this system and in the absence of degradation, all of the proteins should line up on a single diagonal. Here, only the portion of the gel carrying the 68-, 70-, and 84-kDa hsps is shown. The trailing label above the diagonal is due to incomplete solubilization of components for the second dimension. It is clear that degrading activity comigrates with the 70-kDa protein on the basis of size, since the digestion seen in the second dimension must have occurred after the first dimension as with isoelectric focusing (Fig. 2*B*). The foregoing data, taken together, suggested that the 70-kDa protein must be digesting itself, since the putative protease has the same apparent size and isoelectric point.

Since it appeared that the 70-kDa hsp is self-degrading and that this property is shared by each of the multiple forms of the protein, we continued to use two-dimensional separation based on protein size in both directions to study this reaction. This method is similar to that of Cleveland *et al.* (19) except that no protease is added and only self-degrading components are observed. It should be emphasized that in this system all reactions and treatments are carried out within the gel matrix to ensure sterility and retention of high concentrations of components. As shown in Fig. 3, after heat shock at 34°C or 40.7°C and subsequent incorporation of label, electrophoresed samples show a prominent pattern of products apparently derived from the 70-kDa hsp. Although this is by far the most extensive pattern, degradation of the 84-kDa hsp shows faintly here as does fragmentation of a 53-kDa component labeled at 25°C (Fig. 3). On the other hand, the smaller hsps and many other heavily labeled proteins are much less affected. Clearly there is little if any general protease activity in any of the samples.

Repeated Electrophoresis. From a number of experiments it appeared that degradation of the 70-kDa hsp occurs during electrophoresis and for only a short time afterward. That is, a 2-hr incubation after electrophoresis gives the same pattern and amount of products as a 10-hr incubation (data not shown). This suggests that some product inhibits further degradation of the protein and that this inhibitory product is removed by electrophoresis. For a further evaluation of this observation, we made use of successive purifications of the 70-kDa hsp, as illustrated in Fig. 4. Identical aliquots of a crude extract of heat-shocked and labeled wing tissue were electrophoresed in one dimension and then cut out from the wet gel as shown in Fig. 4*A*. A gel block (containing both 70-kDa and 68-kDa hsps) from one lane was incorporated into a new gel and rerun to give the pattern in Fig. 4*B*. The product pattern here is the same as that in Fig. 3 for the proteins from heat-shocked tissue (34°C and 40.7°C). In a similar manner, we have carried samples through as many as five passages with and without trimming the blocks to remove the 68-kDa hsp band. The same pattern was observed at each passage through electrophoresis. Fig. 4*C* shows the result of one such experiment. In this case, the second and third runs are displayed as a diagonal and a vertical pattern. This was achieved by cutting out the whole strip rather than a block after the second passage in one dimension. The strip was then incorporated at right angles in a new gel as were those in Fig. 3. Beside revealing the same product patterns, this experiment also shows that extensive degradation occurs during the 16-hr electrophoresis: (*i*) the diagonal shows degradation components that were produced during the incubation of the gel block from the first dimension before the second electrophoresis; (*ii*) the streaks between the diagonal and the vertical show degradation that occurred during the electrophoresis of the gel block (second electrophoresis); and (*iii*) the vertical pattern shows the degradation that occurred during equilibration of the gel strip, from the second electrophoresis, before the third electrophoresis.

Confirmation that degradation occurs during electrophoresis was obtained by two-thirds reduction of the current to extend the electrophoresis time from the standard 16 hr to 42 hr (Fig. 5); more and smaller degradation products were obtained in the diagonal and cross-connecting stripes. These had to be produced prior to the last equilibration, since only the direct vertical pattern was derived from this last step. The

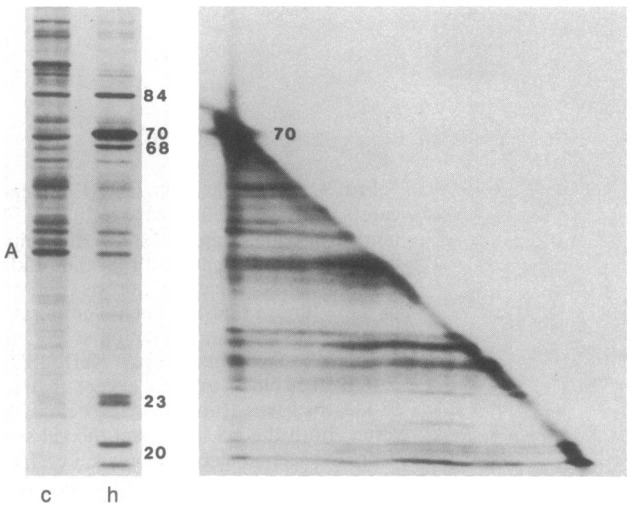

FIG. 5. Degradation during electrophoresis. For reference, one-dimensional $NaDodSO_4$/PAGE of control (c) and heat-shocked (h) samples from *Drosophila* pupal wing tissue is shown at left (A, actin). Two-dimensional $NaDodSO_4$ of the 70-kDa hsp as in Fig. 4*C*, except that the second electrophoresis (first dimension) was carried out at one-third the current used for both dimensions in Fig. 4 *C*; thus, this gel ran for 42 hr rather than 16 hr.

equilibration buffer is neutral with 2% $NaDodSO_4$, whereas the running gel is at pH 8.9 with ≈0.2% $NaDodSO_4$. It appears that degradation occurs under both conditions.

The demonstration of the loss of the 70-kDa hsp *in vivo* was done with larvae, whereas the experiments just described made use of wing tissue from pupae. However, we have carried out similar experiments with larval salivary glands and larval brain and obtained results similar to those obtained with the differentiating wings.

Mammalian Cells. CHO and mouse embryo (L-929) cells both produce hsps of about 70-, 90-, and 100-kDa. For reference, control and heat-shocked CHO cell samples are shown at left in Fig. 6. A rerun in one dimension (see Fig. 4) of the 69- to 70-kDa region as well as the 90- and 100-kDa bands is shown in the center and the third dimension for the 69- to 70-kDa sample is shown at right. In this case the diagonal presents products from both the 69- and the 70-kDa components, whereas the vertical retains the resolution of the two individual bands. These two are obviously very closely related since they give major products with essentially the same spacing. Similar results were obtained with preparations from the mouse cell line L-929. Neither of the mammalian preparations appeared to degrade extensively during electrophoresis as observed for the 70-kDa hsp from *Drosophila*. We have observed also that the 90-kDa hsps of mouse and hamster are relatively stable in this system, whereas the 100-kDa components degrade to an extent similar to those for the 69- and 70-kDa hsps (data not shown).

Non-Heat Shock Proteins. Besides the 70-kDa hsps, we have observed that a number of other proteins from *Drosophila* tissues and mammalian cell lines degrade to various degrees under the same circumstances. An example is shown in Fig. 7. The prominent 130-kDa component that appears in differentiating wing tissue of *Drosophila* at about 75 hr degrades at a relatively high rate. In this experiment the 130-kDa band from the first one-dimensional gel (shown at left) was excised and rerun after only a 30-min equilibration. The strip from the second gel was equilibrated for 6 hr before electrophoresis in the next dimension to give the picture shown at right. In this case most of the original sample was degraded during the 6-hr equilibration at pH 6.8. Degradation during electrophoresis (pH 8.9, 0.2% $NaDodSO_4$) was not evident. The conditions for degradation of this protein are quite different than those for the 70-kDa hsp, showing that this type of degradation is specific for each protein affected.

DISCUSSION

Our initial reaction to the observed instability of the 70-kDa hsp of *Drosophila* (as shown in Fig. 2*B*) suggested involvement of a contaminating protease. Mirault *et al.* (9) invoked a similar suggestion. However, for this to be true, the contaminant would have to move in the same way as the hsp in both isoelectric focusing and sizing ($NaDodSO_4$) gels. That at least three of the isoforms of the 70-kDa hsp show degradation (Fig. 2*B*) makes this explanation even less likely, and we feel that the additional data presented provide strong support for the contention that this protein has the capability for self-digestion. Caizzi *et al.* (11) concluded from a quite different kind of information that the 70-kDa hsp of *Drosophila* could assume different apparent sizes in $NaDodSO_4$ gels. However, this conclusion was based only on fingerprint patterns that were qualitatively similar but quantitatively quite different. On this basis the suggested anomalous behavior of the 70-kDa protein in $NaDodSO_4$ gels remains to be confirmed. It seems more reasonable that these results also involved degradation.

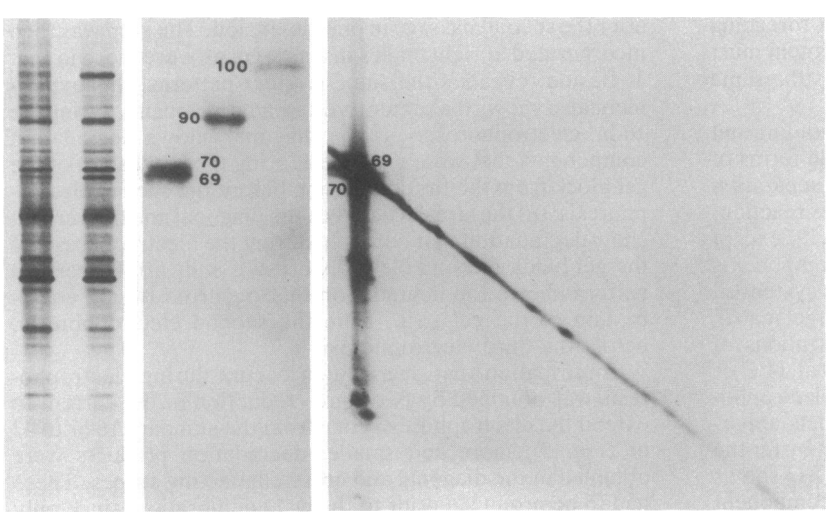

FIG. 6. Degradation of hsps from mammalian cells. Control (c) and heat-shocked (h) samples from the CHO cell line are shown at left in single-dimension $NaDodSO_4$ gels. A rerun of blocks containing the 69- and 70-kDa, the 90-kDa, and the 100-kDa hsps is shown in the center. The third dimension for the 69- plus 70-kDa components is shown by the diagonal and vertical patterns at right. The autoradiogram at right was exposed 36 times as long as that in the center. Thus the degradation products on the diagonal at the right are present but do not show in the shorter exposure in the center.

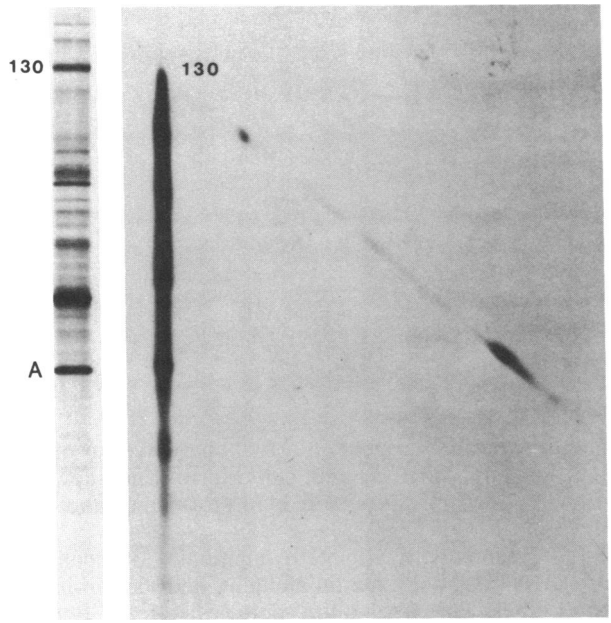

FIG. 7. Spontaneous degradation of a wing protein from *Drosophila*. Shown at left is the pattern of [^{35}S]methionine-labeled proteins from 80-hr pupal wings. The prominent 130-kDa protein is marked, as well as the position of actin (A). Degradation of the 130-kDa protein in the second and third dimensions is shown at right.

In the work presented in this paper, all samples were subjected to rather severe denaturing conditions. All were boiled in 1% $NaDodSO_4$ buffered at pH 6.8. Focused samples were dissolved in 8 M urea, and we have made a number of preparations that have degradation activity after precipitation with trichloracetic acid and drying with alcohol and ether. These various conditions are more than sufficient to inactivate the strong proteolytic action present in extracts of the mouse and hamster cell lines and *Drosophila* larvae that we have used in this work. The wing tissue, in contrast, does not have much endogenous protease activity (see Fig. 2*A*). Thus, it does not appear that we are observing the effects of the normal complement of endogenous proteases. Furthermore, as mentioned earlier, we have retained the 70-kDa hsps and other proteins considered within an acrylamide gel matrix to avoid contamination with proteases from external sources. It is not likely that the gel itself has a catalytic role since, for example, the expected degradation products that should be produced in the gel during electrophoresis (as in Figs. 4 and 5) did not appear in the sample shown in Fig. 2*A*.

For all of these reasons, we feel that the degradation of the 70-kDa hsp and the other proteins considered is derived from self-proteolysis based on the structural potential of each. For example, the amino acid sequence of the 70-kDa hsp from *Drosophila* is known (6) and it contains in the carboxyl end the appropriate arrangements of serine, cysteine, histidine, and aspartic acid residues to become a "serine" type protease if it were folded properly. We have no evidence that this is the case and other kinds of configurations to give protease action are possible. What we are suggesting is that a good many polypeptide chains may have the potential for refolding during or after denaturation, either alone or in combination with a polar/nonpolar component such as $NaDodSO_4$, to yield a product with protease activity. For an *in vivo* situation a role such as that provided by $NaDodSO_4$ *in vitro* could be supplied by fatty acids. These normal components do have specific conformational effects on such diverse proteins as albumin (21) and cytochrome *c* (22).

In any case, we have shown that the 70-kDa hsp specifically decays *in vivo*. *In vitro* there is protease activity associated with the 70-kDa hsp when it is separated from other proteins either on the basis of size or isoelectric point. This activity is limited, seeming to be inhibited by some degradation product that can be removed by electrophoresis. This self-degradation may be a mechanism for the specific decay of the 70-kDa hsp and some other proteins *in vivo*.

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