

Subunit Compositions and Catalytic Properties of Proteasomes from Developmental Temperature-Sensitive Mutants of *Drosophila melanogaster*

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Two dominant temperature-sensitive (*DTS*) *Drosophila* mutants are missense mutations of proteasome genes encoding beta-type subunits $\beta 6/C5$ (*DTS5*) and $\beta 2/Z$ (*DTS7*). At nonpermissive temperature (29°C), heterozygotes (*DTS5/+* and *DTS7/+*) develop normally until metamorphosis; pupae fail to mature and die before eclosion. Proteasomes were purified from wild-type (WT) and heterozygous adult flies raised at permissive temperature (25°C). Two-dimensional gel electrophoresis separated at least 28 proteins, 13 of which were identified with monospecific antibodies to $\alpha 6/C2$ (five species), $\alpha 2/C3$ (three species), $\alpha 7/C8$ (three species), $\alpha 5/zeta$, and $\beta 1/Y$ subunits. Both quantitative and qualitative differences were observed between WT and *DTS/+* proteasomes, with *DTS5/+* deviating more from WT than *DTS7/+* proteasomes. In *DTS5/+* there was a shift to more acidic species of C2 and C3 and a shift to less acidic species of 32-kDa subunits (#3–#7) recognized by an anti-alpha subunit monoclonal antibody (MCP222) and were losses of two 32-kDa subunits (#2 and #3), decreases in Y (25 kDa; 2-fold) and 31-kDa (#9; 2-fold) subunits, and increases in 52-kDa (#1; 1.9-fold) and 24-kDa (#13; 2.3-fold) subunits. In *DTS7/+* there was a less pronounced shift to acidic species of C3 and no pI shift in C2 species and subunits #3–#7 and were decreases in #9 (2.5-fold) and #14 (3-fold) and a loss of #2. The three C8 species were similar between WT, *DTS5/+*, and *DTS7/+* proteasomes. Qualitatively, the most dramatic difference was the appearance of a new 24-kDa subunit (#16) in *DTS/+* preparations, with about a 14-fold greater amount of #16 in *DTS7/+* than in *DTS5/+* proteasomes. Catalytically, WT and *DTS/+* proteasomes had similar peptidase activities, although the *DTS/+* proteasomes were slightly more sensitive to SDS and elevated tem-

peratures *in vitro*. The incorporation of *DTS* subunits apparently altered proteasome assembly and/or processing at permissive temperature with little effect on catalytic activities. These data suggest that at nonpermissive temperature, assembly/processing is more severely affected, producing *DTS*-containing complexes that lack functions essential for cellular proliferation and differentiation at metamorphosis. © 1999 Academic

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Key Words: proteasome; multicatalytic proteinase; *Drosophila*; insect; arthropod.

Ubiquitin/proteasome-dependent proteolysis is a highly conserved system for regulated protein degradation essential for a variety of cellular processes such as skeletal muscle atrophy, programmed cell death, stress response, cell cycle progression, and gametogenesis, fertilization, and development [see (1–3) for reviews]. The 26S proteasome, composed of a 20S proteasome or multicatalytic proteinase (MCP)² and two PA700 ATPase regulatory complexes, degrades proteins targeted via the covalent binding of multiubiquitin chains by ubiquitin conjugases.

The 20S proteasome consists of a cylindrical-shaped particle composed of four heteroheptameric rings (two outer alpha and two inner beta) enclosing two antechambers and a central catalytic chamber (4). The alpha rings serve a regulatory function by binding PA700

² Abbreviations used: Boc, *t*-butyloxycarbonyl; BraAP, branched-chain amino acid-preferring; Cbz, benzyloxycarbonyl; DMSO, dimethyl sulfoxide; *DTS*, dominant temperature-sensitive; ECL, enhanced chemiluminescence; MCP, multicatalytic proteinase (EC 3.4.99.46); β NA, β -naphthylamide; pAB, *p*-aminobenzoate; pNA, *p*-nitroanilide; PGPH, peptidylglutamylpeptide hydrolase; PVDF, polyvinylidene difluoride; SNAAP, small neutral amino acid-preferring; TBS, Tris-buffered saline; TTBS, Tween 20 in TBS; WT, wild-type.

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and other regulators and undergoing posttranslational modifications that affect intracellular location and catalytic activities, whereas the beta rings, either directly or indirectly, serve a catalytic function (1). Six distinct peptidase activities have been measured, although the trypsin-like, neutral chymotrypsin-like, and PGPH activities are better characterized than the acidic chymotrypsin-like, BrAAP, and SNAAP activities (1). Three of the 7 beta subunits (X or $\beta 5$, Y or $\beta 1$, and Z or $\beta 2$) have N-terminal threonines essential for proteolytic activity (4–6). The 14 subunits occupy specific positions, forming a complex dimer around a single axis of symmetry (4, 7). The yeast topology is the basis of the $\alpha 1$ -7/ $\beta 1$ -7 nomenclature which assumes that the subunit positions are invariant in eukaryotes (4, 8).

In *Drosophila*, 2 of 10 dominant temperature-sensitive (*DTS*) lethal mutants, originally described by Holden and Suzuki (9), are missense mutations of genes encoding beta subunits homologous to mammalian C5 [*DTS5* or *1(3)73Ai¹*] and Z (*DTS7*) subunits (10, 11). These mutations result in the replacement of threonine with isoleucine at position #18 in *DTS5* (position #47 before autolytic processing) and glycine with arginine at position #170 in *DTS7* (#209 before processing). Both Thr18 and Gly170 are conserved in homologous subunits of such divergent organisms as mammals and yeast (10, 11). The mutants have similar phenotypes and interact genetically (9, 11). Heterozygotes with either mutant allele fail to survive to adulthood when raised at elevated temperature (29°C), with development reasonably normal until metamorphosis. In pupae imaginal disc derivatives are reduced, head eversion often fails, and adult abdominal segments do not develop; pupae die before eclosion. Both mutants behave genetically as dominant negative mutations, since heterozygous deletions of either locus have no phenotypic effect and since the *DTS* phenotype of either *DTS5/+* or *DTS7/+* can be rescued by an extra copy of the wild-type gene (10, 11). These data suggest that the *DTS* alleles encode "poison subunits," which, when incorporated into the complex, render the complex poorly functional.

The *DTS* alleles could be useful in elucidating the role of the proteasome and ATP/ubiquitin-dependent proteolysis in *Drosophila* development, during which changes in proteasome immunocytochemical localization and subunit composition occur (12, 13). This study examined the effects of *DTS* subunits on the subunit compositions and catalytic properties of *Drosophila* proteasomes. Enzymes were isolated from adult WT (+/+) and *DTS/+* heterozygotes raised at permissive temperature. This facilitated collection of sufficient numbers of mutant individuals for analysis, since raising heterozygotes at nonpermissive temperature would have restricted collection to dying larvae or pupae. Furthermore, *DTS/DTS* flies die at 25°C, suggesting that mutant subunits are not perfectly normal even at

permissive temperature. Subunit compositions were analyzed with two-dimensional PAGE. Five peptidase activities were measured, and the effects of proteinase inhibitors, SDS, and temperature were determined. The *DTS/+* proteasomes retained catalytic function despite alterations in subunit composition. A hypothetical model is presented showing how incorporation of *DTS* subunits at nonpermissive temperature could produce nonfunctional complexes.

MATERIALS AND METHODS

Animals. Wild-type (+/+) and *DTS/+* *Drosophila melanogaster* flies were raised to adults at permissive temperature (25°C) as described (10).

Chemicals. Boc-VEAL-NA and Cbz-IE(Ot-Bu)AL-CHO were provided by Dr. Sherwin Wilk, Mount Sinai School of Medicine; Cbz-GPALG-pAB and Cbz-GPFL-CHO were provided by Dr. Marian Orłowski, Mount Sinai School of Medicine; CEP-1508 was provided by Dr. Robert Siman, Cephalon Inc.; monoclonal antibodies [MCP71 (anti-zeta), MCP236 (anti-C3), MCP106 (anti-C2), MCP222 (anti-alpha), and MCP72 (anti-C8)] were provided by Dr. Klavs Hendil, University of Copenhagen; and an anti-Y polyclonal antibody was provided by Dr. John Monaco, University of Cincinnati. Cbz-GGR- β NA, Cbz-GGL-pNA, Cbz-LLE- β NA, and aminopeptidase-N were purchased from Sigma; ampholines (Bio-Lyte) 5/7 and 3/10 and nitrocellulose were from Bio-Rad; PVDF membrane (Hybond-P) was from Amersham; glutaraldehyde was from Kodak; Vectastain ABC kit was from Vector Laboratories; and ECL Western blotting detection kit was from Amersham. All other chemicals used were at least reagent grade and were purchased from Sigma, Mallinckrodt, EM Science, Bio-Rad, or Fisher Scientific.

Enzyme purification. Proteasomes were purified from adult flies using the same procedure as that used for the purification of proteasome from the body wall of hawkmoth *Manduca sexta* larvae (14). Adult flies were frozen and stored at -80°C until needed. Twenty grams of whole flies was homogenized in 6.7 vol of buffer A (20 mM Tris-acetate, pH 7.5, 20 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol) and centrifuged at 16,300g for 20 min. Protein in the supernatant fraction was precipitated between 45 and 65% saturated ammonium sulfate and chromatographed on arginine-Sepharose, HPLC Mono Q (Pharmacia) anion-exchange (15), and HPLC TSK-phenyl-5-PW (Bio-Rad) hydrophobic interaction columns (14). Protein concentration was determined by fluorescence emission spectroscopy (16). At least three different preparations were made of each genotype.

Two-dimensional PAGE analysis and Western blotting. Purified *Drosophila* proteasomes were separated by two-dimensional PAGE (17). Two different preparations from WT flies and three each from *DTS5/+* and *DTS7/+* flies were analyzed. Initially, gels were either stained with silver (18) or transferred to nitrocellulose (19). After a brief staining with Ponceau S to locate subunits, membranes were blocked with 3% gelatin in TBS (1 h) and incubated with monoclonal antibody (MCP71 at 1:1000 dilution; MCP236 at 1:5000 dilution; MCP222 at 1:1000 dilution; or MCP106 at 1:1000 dilution in TTBS) for 1 h as described (14). Membranes were then incubated with biotinylated rabbit anti-mouse IgG (1:10 normal concentration or 1:2000 dilution) in TTBS for 2 h. Detection was accomplished by a 30-min incubation with Vectastain ABC reagent (1:10 normal concentration or 1:500 dilution), followed by chemiluminescent detection with ECL reagent (14).

In subsequent analyses, protein was transferred to PVDF membrane and stained with colloidal gold before blocking with 5% nonfat milk in TBS. This allowed direct comparison of exposed films with proteins stained with colloidal gold for unambiguous identification of subunits (20). Blots were incubated with polyclonal anti-Y antibody

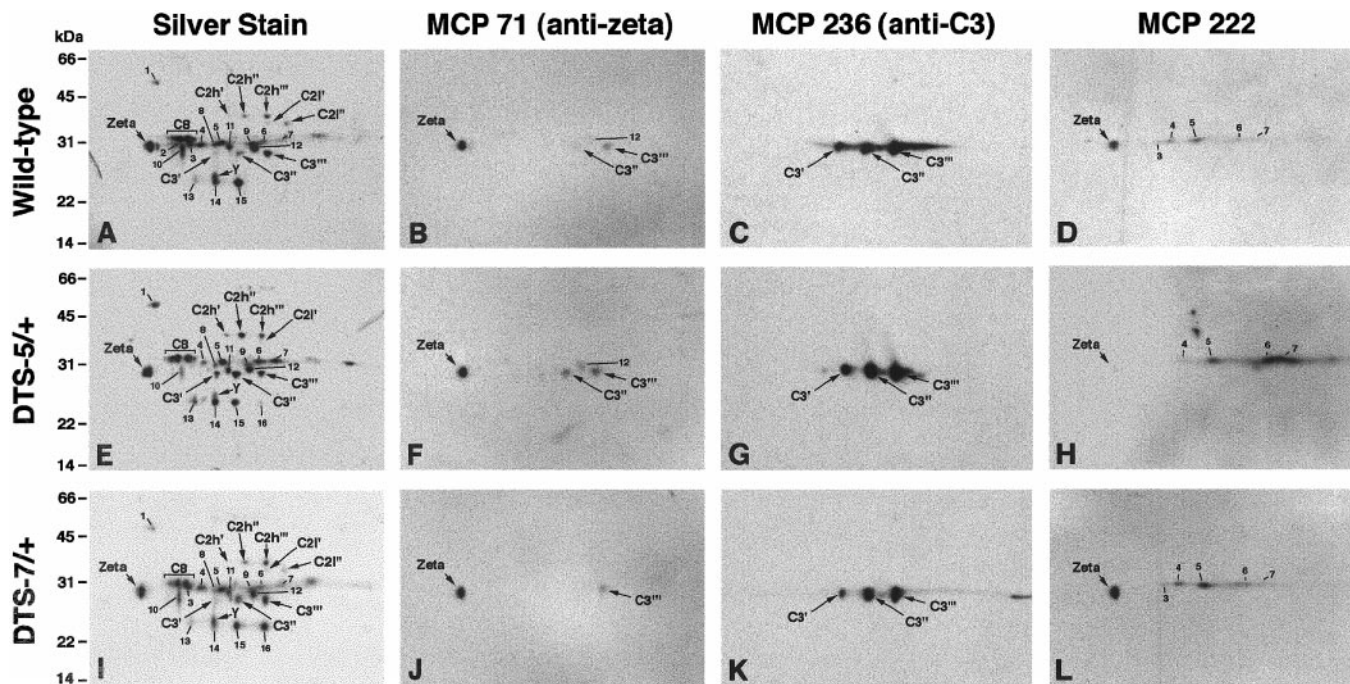


FIG. 1. Two-dimensional gel and immunoblot analysis of WT (+/+) and DTS/+ *Drosophila* proteasomes (5 μ g) purified from flies raised at permissive temperatures. Gels were stained with silver (A, E, and I) or transferred to nitrocellulose and probed with monoclonal antibodies specific for the zeta subunit (B, F, and J), C3 (Pros25) subunit (C, G, and K), or an anti- α -subunit epitope in zeta and subunits #3–#7 (D, H, and L). DTS/+ subunit patterns differed from the WT condition, with DTS5/+ showing greater deviation than DTS7/+ proteasome. In DTS5/+, there was a shift to more acidic species of higher molecular mass forms of C2 (C2h' and C2h''); (E) and C3 (C3' and C3''); (E and G), a decrease in lower molecular mass forms of C2 (C2l' and C2l''); (E), and a shift to less acidic subunits (#6 and #7) recognized by the MCP222 antibody (E and H). There was also a new subunit (#16) found in small amount in DTS5/+ (E) but in greater amount in DTS7/+ (I). DTS7/+ showed a small shift to more acidic C3 species (C3', C3''); (E and I). Densitometric measurements of A, E, and I (standardized to the staining of the zeta subunit) are presented in Table II. Major differences from WT are summarized in Fig. 3. The gels and blots are oriented with the acidic end on the left.

(1:50,000; 1 h) followed by biotinylated goat anti-rabbit IgG (1:10 normal concentration; 1 h) and ABC reagent (1:10 normal concentration; 30 min). The same blots were stripped with 2% SDS, 100 mM β -mercaptoethanol, and 67.5 mM Tris-HCl (pH 6.7) at 60°C for 30 min and reprobed with mouse monoclonal MCP 72 (1:1,000), followed by biotinylated anti-mouse IgG and ABC reagent as above.

Densitometry. Scanning densitometry was conducted on the silver-stained gels shown in Figs. 1A, 1E, and 1I. Each image was acquired using a cooled CCD Nikon Nikor box camera (Technology Resources) equipped with a 50-mm f.8AF lens, Photometrics CE200A Camera Electronics unit, and PMIS-S200 image-processing software (version 3.5.4 VC 1.0-S200.C; Gregory K. Remington; distributed by GKRCC). Image analysis was conducted with Phototrix 2D Full Gel Analysis software (release 3.51; Nonlinear Dynamics Ltd.; distributed by Photometrics Inc.). Density of spots was determined by measuring pixel volume of the recorded image. Background subtracted from the image was determined by a variable method consisting of the random selection and averaging of six background locations. The standard error for pixel volume readings was obtained by averaging values obtained from four separate variable background subtractions. This method resulted in a variance of 0.4–3.0% in pixel volume for recorded spots, with the larger variances generally associated with the smaller spots. As long as an entire spot was included in the area selected for volume analysis, the size of the area selected did not significantly affect pixel volume. This indicated that the background had no significant effect on pixel volume. Spots on different gels were quantitatively compared by calculating a relative pixel volume based on the zeta subunit (relative pixel volume = pixel volume/zeta pixel volume). The zeta subunit was selected as a refer-

ence because it was relatively constant between preparations and gels (e.g., subunit volumes in Fig. 1 were 12,719 for WT, 13,196 for DTS5/+, and 13,231 for DTS7/+).

Peptidase assays. Trypsin-like, PGPH, and acidic chymotrypsin-like activities were measured using Cbz-GGR- β NA, Cbz-LLE- β NA, and Boc-VEAL-NA, respectively. Neutral chymotrypsin-like and BrAAP activities were measured using Cbz-GGL-pNA and Cbz-GPALG-pAB, respectively. Unless otherwise indicated, reaction conditions were the same as described previously (21, 22). Inhibitors were dissolved in DMSO. An equal volume of DMSO was added to all control reactions for assays involving these inhibitors. Fluorometric detection was used to measure β NA released by peptide hydrolysis (21). Free pNA and pAB were diazotized and measured spectrophotometrically at 540 nm using a Perkin-Elmer Lambda Bio UV/VIS spectrophotometer (22). Activities are presented as μ mol peptide hydrolyzed/mg MCP/h.

RESULTS

Purification and yields. The chromatography procedure resulted in highly purified proteasomes, as shown previously for proteasome from hawkmoth larval tissues (14). Activity coincided with a single protein peak in one to two fractions eluting from the final column (data not shown). The yields were consistently about 1.3 mg enzyme from 20-g adult flies regardless of genotype, indicating that DTS subunits were being

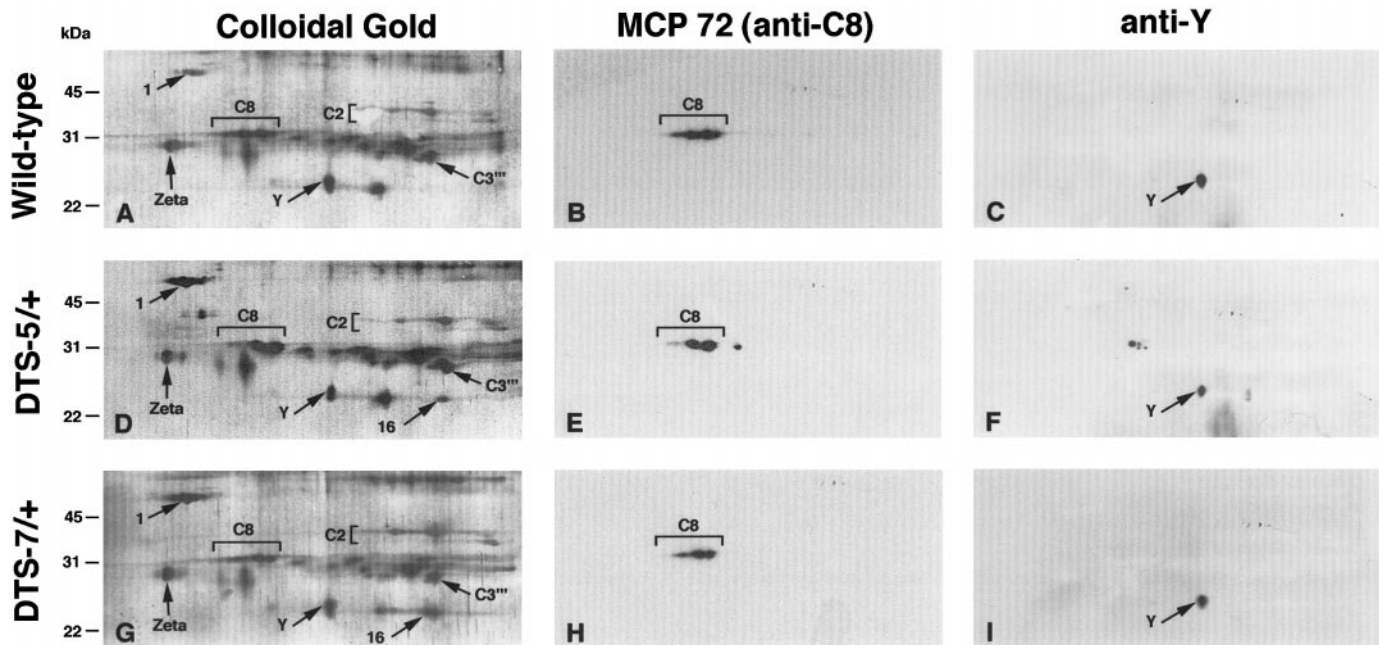


FIG. 2. Two-dimensional immunoblot analysis of WT (+/+) and DTS/+ *Drosophila* proteasomes (10 μ g) purified from flies raised at permissive temperature. Proteins in gels resolving the more acidic species were transferred to PVDF membrane and stained with colloidal gold (A, D, and G). Stained blots were probed with antibody to C8 subunit (B, E, and H) and stripped and reprobed with antibody to the Y (δ) subunit (C, F, and I) (see Materials and Methods). There was a reduction of the Y subunit in DTS5/+ (F), but the relative amounts of the three C8 species were similar in preparations from the three genotypes (B, E, and H). Resolution of C8 was reduced in these blots because the amount of protein loaded on the gels was doubled to 10 μ g (compare with Figs. 1A, 1E, and 1I, in which 5 μ g was used). The reduced amount of the most acidic C8 species in E was not observed in two other proteasome preparations from DTS5/+ flies (see Fig. 1E). The "spot" to the right of C8 in E is an artifact on the film. The blots are oriented with the acidic end on the left.

expressed and incorporated into complexes. The results are representative of electrophoretic and catalytic analyses from two or three different enzyme preparations of each genotype.

TABLE I

Calculated Masses and *pI*s of *Drosophila* 20S Proteasome Subunits That Have Been Cloned and Sequenced [(10, 11, 29–33), J. M. Belote and K. A. Smyth, Unpublished Observations]

20S subunit	Name	Mass (kDa)	<i>pI</i>	Accession No.
α 1	α 1 (C7, iota)	27.1	7.55	GenBank AF144749
α 2	Pros25 (C3)	25.9	6.15	SwissPro P40301
α 3	Pros29 (C9)	29.4	7.74 ^b	SwissPro P18053
α 4	Pros28.1 (C6-I)	28.0	8.30 ^b	SwissPro P22769
	Pros28.1A (α 4t1)	27.9	9.12 ^b	GenBank U46008
	Pros28.1B (α 4t2)	28.8	6.32 ^b	GenBank U46009
α 5	Zeta	26.8	4.60	SwissPro Q95083
α 6	Pros35 (C2)	31.0	6.00	SwissPro P12881
α 7	C8	27.4	5.58	GenBank AF025793
β 2	Z (DTS7)	25.4 ^a	7.94 ^{a,b}	GenBank AF025792
β 3	C10-II	23.2	5.01	GenBank AF116898
β 6	C5 (DTS5)	23.5 ^a	5.50 ^a	GenBank U00790

^a After autocatalytic processing that removes N-terminal prosequence.

^b Includes cysteine residues.

Subunit composition of wild-type proteasome. Two-dimensional PAGE of WT proteasome showed a complex pattern of at least 28 proteins (Figs. 1A and 2A). Thirteen of these were identified with monospecific antibodies. Only zeta (α 5) and Y (β 2) appeared as single species (Figs. 1B and 2C). Subunit C3 (Pros25 or α 2) occurred as three forms of similar mass (30 kDa) differing in net charge (Fig. 1C). Subunit C8 (α 7) also occurred as three forms of similar mass (32 kDa; Figs. 1A and 2B). There were five species of subunit C2 (Pros35 or α 6) identified by monoclonal antibody MCP106 (data not shown): three higher molecular weight forms (38 kDa; C2h) and two lower molecular weight forms (35 kDa; C2l) (Fig. 1A). MCP222, a monoclonal antibody that recognizes an epitope in some alpha-type subunits (14), reacted with zeta (30 kDa) and five (#3–#7) 32-kDa subunits (Fig. 1D). Since subunits #3–#7 reacted with the same antibody had the same mass and had similar total relative pixel volumes (161, 195, and 188 for WT, DTS5/+, and DTS7/+, respectively), they may be differentially modified products of the same gene. The anti-zeta antibody reacted weakly with C3 and subunit #12 (Figs. 1B, 1F, and 1J).

The estimated masses and *pI* values of *Drosophila* proteasome subunits that have been cloned and sequenced are presented in Table I. The positions of identified subunits (zeta, C2, C3, and C8; the Y subunit

TABLE II
Scanning Densitometry of *Drosophila* Proteasome Subunits

Subunit	kDa	Wild type	DTS5/+		DTS7/+	
		Relative volume ($\times 10^{-2}$)	Relative volume ($\times 10^{-2}$)	% of wild-type	Relative volume ($\times 10^{-2}$)	% of wild-type
1	52	18	42	233	12	67
C2h'	38	^a	^a	NA	^a	NA
C2h''	38	11	40	364	13	118
C2h'''	38	21	25	119	26	123
C21'	35	^a	^a	NA	^a	NA
C21''	35	11	2	18	7	64
2	32	17	0	0	0	0
3	32	29	0	0	23	79
4	32	48	20	42	53	110
5	32	41	56	139	56	137
6	32	26	59	227	29	112
7	32	17	60	353	27	159
9	31	52	27	52	21	40
C3'	30	7	29	414	11	161
C3''	30	24	65	271	47	196
C3'''	30	58	44	76	62	107
Y	25	32	15	47	45	141
13	24	16	30	188	13	81
14	24	38	54	138	13	33
15	24	78	48	62	53	68
16	24	0	5	NA	69	NA

Note. Scanning densitometry was conducted on gels shown in Figs. 1A, 1E, and 1I. Data are expressed in relative pixel volume ($\times 10^{-2}$) and as a percentage of WT (+/+). Relative pixel volume was obtained by dividing the pixel volume of the subunit in question by the pixel volume of the zeta subunit on the same gel. Standard error for pixel volume values was between 0.4 and 3.0% of the relative volume recorded (see Materials and Methods). Data for subunits #8, #10, #11, and #12 and the C8 family were not included because they did not differ between WT and DTS/+ proteasomes. NA, not applicable.

^a Amounts were too low to measure accurately.

sequence is not in the database) were consistent with these estimates, except that they had a lower electrophoretic mobility (i.e., greater molecular masses) in the second dimension than that expected from the deduced amino acid sequence. It is also apparent that the pH gradient established with equilibrium isoelectric focusing in the first dimension did not resolve the more basic subunits, such as Pros29 (C9), Pros28.1, and Z (Table I; Figs. 1 and 2), unless posttranslational modifications lowered their *pI* values so that they migrated to the acidic-neutral range of the pH gradient. Since the basic *Drosophila* subunits are better resolved under non-equilibrium conditions (13, 23, 24), it is likely that these subunits did not enter the gel and are consequently not included in the analysis. Also, our analysis may not have detected the expected increase in *pI* of the mutant Z (DTS7) subunit.

Unfortunately, antibodies that recognize the *Drosophila* C5 and Z subunits were not available for this study and thus we were not able to identify the two subunits that could be the most affected by the DTS mutations. The anti-C5 antibody raised against *Drosophila* (10) lost specificity after the freezer in which it was stored failed. Several attempts using different

combinations of protocols and detection methods were unsuccessful.

Subunit compositions of DTS/+ proteasomes. Even with the limitations of the analysis given above, qualitative and quantitative differences between WT and DTS/+ proteasomes were observed. Proteins in silver-stained gels (Figs. 1A, 1E, and 1I) were quantified by scanning densitometry and the resulting pixel volumes were standardized to the zeta subunit to control for small differences in loading (Table II). Zeta was selected as a reference because it migrated as a single species well-separated from other subunits and it did not vary between WT and DTS/+ preparations. The major changes (>1.5-fold) from WT are summarized in Fig. 3. A complete densitometric analysis was done on one preparation from each genotype, although two-dimensional PAGE analyses (Fig. 2 and data not shown) of other preparations (one from WT and two each from DTS5/+ and DTS7/+) were consistent with the results presented in Table II and Fig. 3.

The DTS5/+ two-dimensional PAGE pattern differed more from the WT pattern than DTS7/+. The lower molecular mass species of C2 (C21' and C21'') decreased while

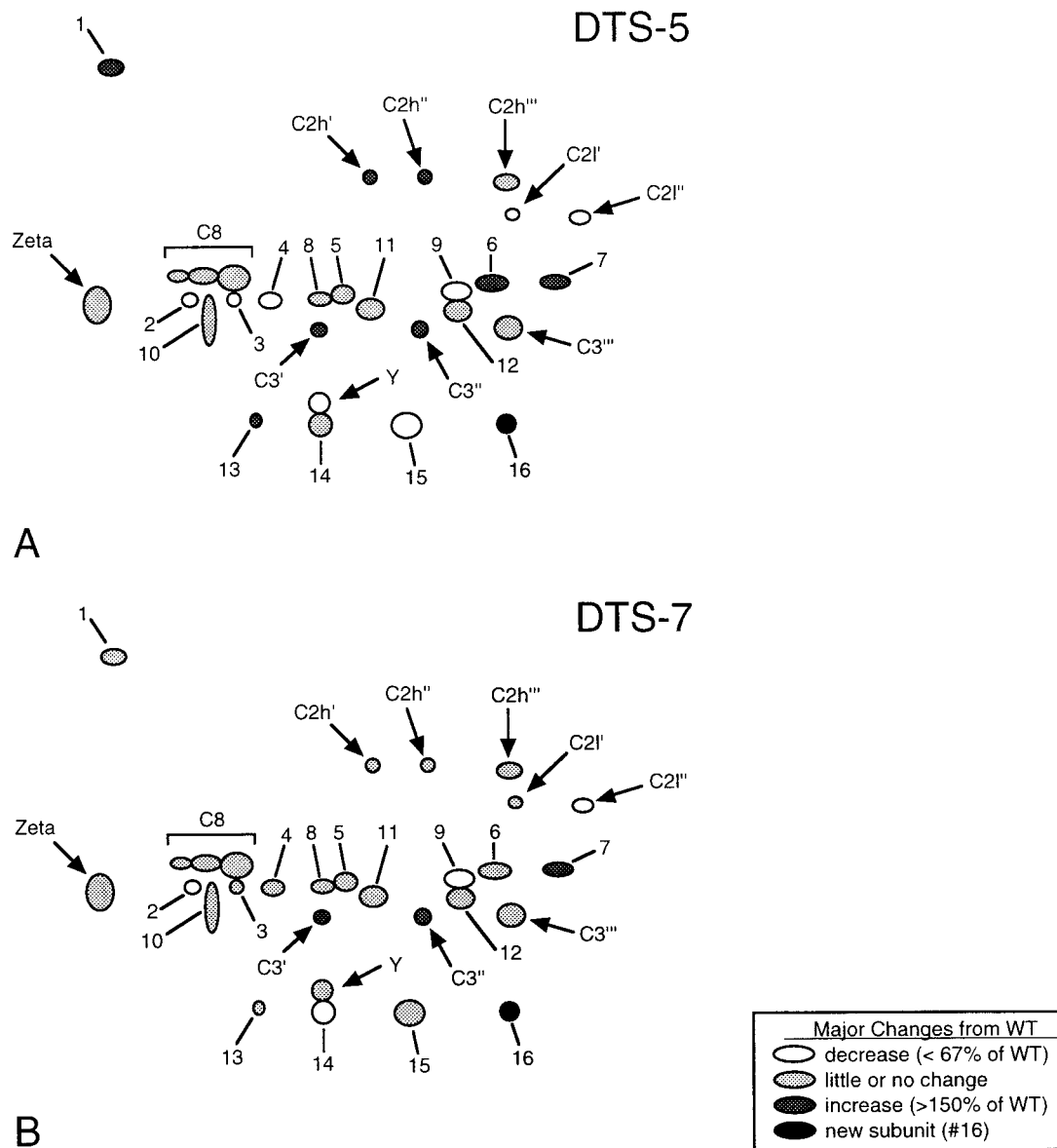


FIG. 3. Diagram summarizing the major changes in subunit patterns of DTS/+ proteasomes. Data are from Figs. 1 and 2 and Table II. A major change is defined as that being greater than a 1.5-fold difference (<67 or >150%) from WT. A new 24-kDa subunit (#16) was present in DTS/+ complexes, with a greater amount in DTS7/+ than in DTS5/+ proteasomes.

the more acidic higher molecular mass species (C2h' and C2h'') increased (Fig. 1E; Table II). There was also a large shift to more acidic species of C3 (C3' and C3''). In DTS5/+ there was a loss of #3, a decreased amount of #4, and increased amounts of #6 and #7 (Fig. 1H). This shift to less acidic species did not occur in either WT or DTS7/+ proteasomes (Figs. 1D and 1L). There were decreases in Y (2.1-fold), #9 (1.9-fold), and #15 (1.6-fold), increases in #1 (2.3-fold) and #13 (1.9-fold), and a complete loss of #2 in DTS5/+ proteasomes (Table II; Figs. 1E and 2F). The relative amounts of the three C8 species were the same in all three proteasome preparations (Figs. 2B, 2E, and 2H). Other subunits in the DTS5/+

proteasome that did not differ substantially from WT were C2h''', C3''', #5, #8, #10–#12, and #14.

DTS7/+ proteasomes had fewer quantitative differences from WT (Table II). The distributions of the five C2 species and subunits #3–#7 were essentially the same as WT, except for moderate reductions in #3 (1.3-fold) and C2l'' (1.6-fold) and a moderate 1.6-fold increase in #7 (Figs. 1I and 1L). There was a shift to the more acidic C3 species, but the increases in C3' (1.6-fold) and C3'' (2-fold) were not as large as those observed in DTS5/+ (Table II; Figs. 1I and 1K). The major difference was the presence of a new 24-kDa subunit (#16), which was also present in smaller

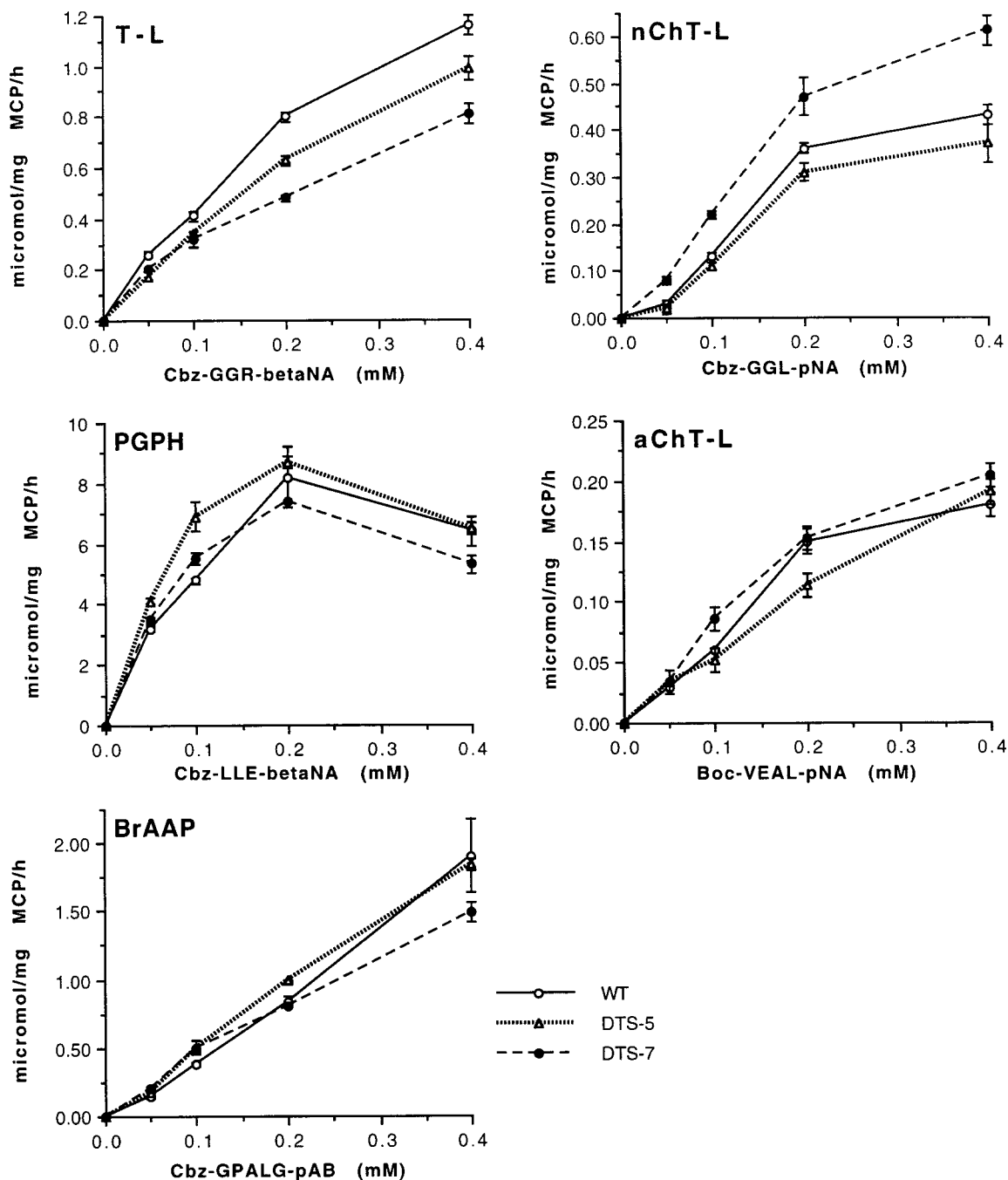


FIG. 4. Peptidase activities of WT (+/+) and DTS/+ *Drosophila* proteasomes as a function of substrate concentration. Unless otherwise noted, reaction mixtures contained 20 mM Hepes-NaOH (pH 8) and 1 μ g proteasome (see Materials and Methods). Peptidase activities measured were trypsin-like (T-L; 3 h incubation), neutral chymotrypsin-like (nChT-L; 6 h incubation), PGPH (0.006% SDS; 1 h incubation), acidic chymotrypsin-like (aChT-L; 2 μ g proteasome in 25 mM Tris-acetate, pH 6; 3 h incubation), and BrAAP (2 μ g proteasome; 0.005% SDS; 3 h incubation). Activities are expressed as means \pm 1 SE ($n = 3$); the SE is not indicated if less than 0.01.

amount in DTS5/+ (Table II; Figs. 1E and 1I). As in DTS5/+, there was a loss of #2 and a decrease in #9 (2.5-fold) in DTS7/+ (Fig. 1I). The other subunits, including Y, C3^{'''}, and C8, showed little or no change from WT, except for a 3-fold decrease in #14 (Table II; Figs. 1I, 2I, and 3).

Peptidase activities. All three proteasome preparations hydrolyzed substrates for five peptidase activities: trypsin-like (T-L), neutral chymotrypsin-like (nChT-L), PGPH, acidic chymotrypsin-like (aChT-L), and BrAAP activities (Fig. 4). *Drosophila* proteasomes have low SNAAP activity (25) and therefore it was not

TABLE III
Effects of Proteinase Inhibitors Cbz-IE(Ot-Bu)AL-CHO, CEP-1508, and Cbz-GPFL-CHO on Peptidase Activities of WT (+/+) and DTS/+ *Drosophila* Proteasomes

Activity	WT		DTS5/+		DTS7/+	
	$\mu\text{mol/mg/h}$	% control	$\mu\text{mol/mg/h}$	% control	$\mu\text{mol/mg/h}$	% control
A. Cbz-IE(Ot-Bu)AL-CHO						
Trypsin-like	0.30 \pm 0.05	41	0.58 \pm 0.01	57	0.64 \pm 0.02	60
Neutral chymotrypsin-like	0.03	9	0.02	4	0.04	6
Acidic chymotrypsin-like	0.02	15	0.02	14	0.02	10
PGPH	2.67 \pm 0.70	93	4.47 \pm 0.59	130	3.30 \pm 0.40	60
BrAAP	1.50 \pm 0.03	79	2.07 \pm 0.06	91	1.57 \pm 0.03	71
B. CEP-1508						
Trypsin-like	0.53 \pm 0.05	73	0.76 \pm 0.01	75	0.67 \pm 0.04	63
Neutral chymotrypsin-like	0	0	0	0	0	0
Acidic chymotrypsin-like	0.02	15	0.02	14	0.02	10
PGPH	0	0	0	0	0	0
BrAAP	0	0	0	0	0	0
C. Cbz-GPFL-CHO						
Trypsin-like	1.06 \pm 0.06	145	1.52 \pm 0.02	149	1.84 \pm 0.01	172
Neutral chymotrypsin-like	0.30 \pm 0.01	105	0.48 \pm 0.02	106	0.61 \pm 0.03	100
Acidic chymotrypsin-like	0.18	156	0.21 \pm 0.01	169	0.25 \pm 0.01	140
PGPH	0.40	14	0.37 \pm 0.09	11	0.55 \pm 0.12	10
BrAAP	0.05	3	0.08 \pm 0.01	3	0.05	2
D. Control (no inhibitors)						
Trypsin-like	0.73		1.02 \pm 0.01		1.07 \pm 0.08	
Neutral chymotrypsin-like	0.29 \pm 0.03		0.45 \pm 0.02		0.61 \pm 0.01	
Acidic chymotrypsin-like	0.12		0.12 \pm 0.01		0.18 \pm 0.01	
PGPH	2.87 \pm 0.09		3.43 \pm 0.20		5.47 \pm 0.18	
BrAAP	1.91 \pm 0.03		2.29 \pm 0.07		2.21 \pm 0.12	

Note. Activities were measured as described under Materials and Methods with 10 μM inhibitor in 20 mM Hepes-NaOH (pH 8) unless otherwise noted. Reactions were preincubated for 10 min prior to the start of the reaction by the addition of substrate. PGPH and BrAAP activities were activated with 0.006 and 0.004% SDS, respectively. Acidic ChT-like activity was measured in 25 mM Tris-acetate (pH 6). Activities are expressed as means \pm 1 SE ($n = 3$) and as a percentage of control (D); the SE is not indicated if less than 0.01.

assayed. The same preparations were used for the five substrates to compare the different activities. The aChT-L and BrAAP activities were similar between WT and DTS/+ proteasomes. The T-L activity of DTS/+ proteasomes was less than that of WT in the preparations shown in Fig. 4, but other DTS/+ preparations showed similar or greater activity against the T-L substrate as WT (e.g., see Table III and legend to Fig. 6). In the preparations used for the inhibitor experiments (Table III), the PGPH activity of WT complexes is less than that of DTS/+ complexes, but this difference was not observed in other preparations (Figs. 4 and 5). However, the nChT-L activity of the DTS7/+ proteasome was consistently greater than that of WT or DTS5/+ (see also Table III), although the relative magnitude varied between preparations.

The effects of three proteinase inhibitors on the five peptidase activities are presented in Table III. Cbz-IE(Ot-Bu)AL-CHO preferentially inhibited the nChT-L and aChT-L activities of *Drosophila* proteasomes. Cbz-

GPFL-CHO preferentially inhibited the PGPH and BrAAP activities and stimulated the T-L and aChT-L activities. CEP-1508, a dipeptide aldehyde (26), strongly inhibited the aChT-L, nChT-L, PGPH, and BrAAP activities. The differences between means were analyzed, using a Student *t* distribution with a 95% confidence interval and pooled *df*, to determine the statistical significance of differences in inhibitor sensitivity between WT and DTS/+ proteasomes. This analysis revealed a greater sensitivity of WT T-L activity to Cbz-IE(Ot-Bu)AL-CHO than the T-L activity of DTS/+ complexes. Cbz-IE(Ot-Bu)AL-CHO also had a greater inhibitory effect on the BrAAP activity of WT and DTS7/+ proteasomes than on DTS5/+. There were no significant differences in the effects of CEP-1508 and Cbz-GPFL-CHO on the peptidase activities between WT and DTS/+ preparations.

Both the PGPH and BrAAP activities of *Drosophila* proteasomes were activated by low concentrations of SDS (Fig. 5). The aChT-L activity was also stimulated

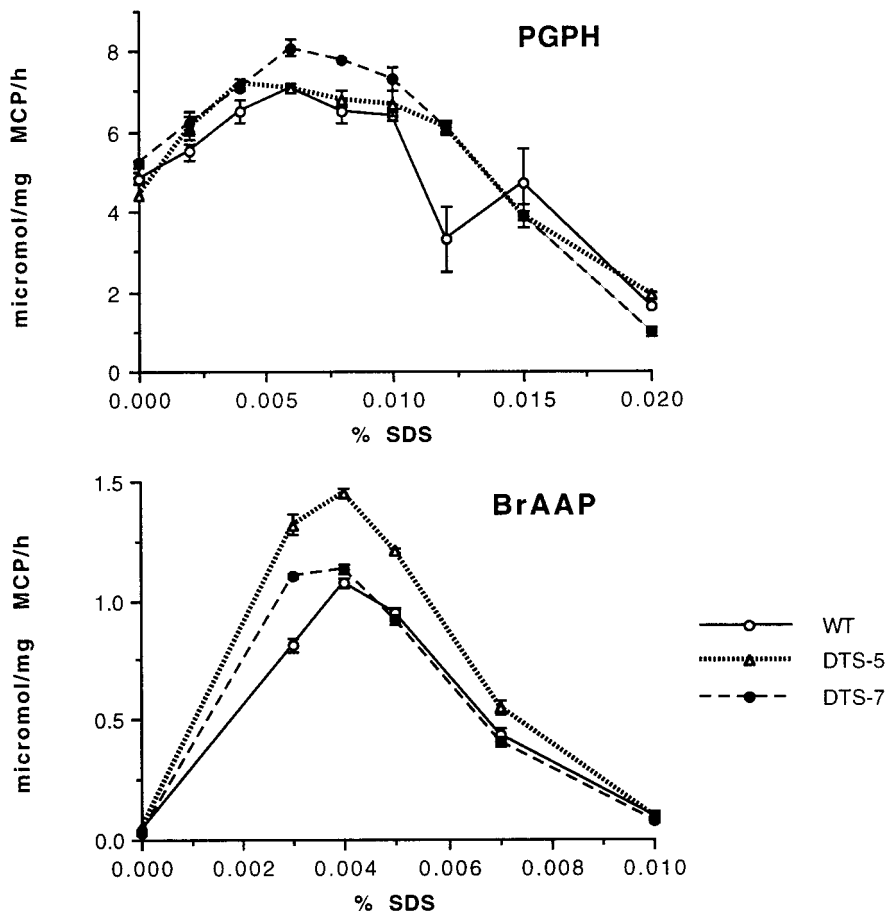


FIG. 5. Effects of SDS on PGPH (top) and BrAAP (bottom) activities of WT (+/+) and DTS/+ *Drosophila* proteasomes. PGPH reaction mixtures were incubated for 1 h at room temperature with 0.4 mM Cbz-LLE- β NA and 1 μ g proteasome in 20 mM Hepes-NaOH (pH 8). BrAAP reaction mixtures were incubated for 3 h at room temperature with 0.2 mM Cbz-GPALG-pAB, 2.1 μ g proteasome, and aminopeptidase-N (0.26 U/ml) in 20 mM Hepes-NaOH (pH 8). SDS had similar effects on PGPH activity of WT and DTS/+ proteasomes. BrAAP activity of DTS/+ proteasomes was more sensitive to SDS than that of WT proteasome. Activities are expressed as means \pm 1 SE ($n = 3$); the SE is not indicated if less than 0.01.

(data not shown). Unlike mammalian and other arthropod proteasomes (25), *Drosophila* proteasome had substantial PGPH activity in the absence of SDS. The response of PGPH activity was similar between WT and DTS/+ complexes, with maximal activation (about 50%) at 0.006% SDS (Fig. 5, top). The BrAAP activity of DTS/+ proteasomes, however, was more sensitive to SDS. Although maximal activation occurred at 0.004% SDS for all three preparations, the BrAAP activity of DTS/+ complexes at 0.003% SDS was greater than that of WT (Fig. 5, bottom).

Since the DTS alleles produce a temperature-sensitive phenotype, the thermal stabilities of WT and DTS/+ proteasomes were examined. Enzymes were preincubated for 1 h at 21, 30, 37, 43, or 50°C and then assayed for T-L activity using a 1-h incubation at the preincubation temperature. *Drosophila* proteasomes were remarkably heat-stable, although DTS/+ proteasomes were more sensitive to elevated temperatures than WT complexes (Fig. 6).

DISCUSSION

Two-dimensional gel analysis of the *Drosophila* 20S proteasome shows the numbers of proteins in excess of the likely number of genes encoding proteasome subunits. At least 28 proteins of varying abundance are observed [Figs. 1 and 2; see also (12, 13, 27)]. The actual number is greater, since isoelectric focusing did not resolve subunits with more basic pI values. There are multiple species of C2, C3, and C8 (Figs. 1 and 2). Similar patterns are also found in proteasomes from lobster and hawkmoth tissues (14, 15, 28). Since a single particle contains two copies of each of the 14 subunits, the proteasome preparations obtained from adult flies are heterogeneous in composition.

Proteasome heterogeneity may result from a combination of the expression of gene variants encoding subunit isoforms, isoforms generated by alternative mRNA splicing, and posttranslational modifications in different tissues and even in different locations within

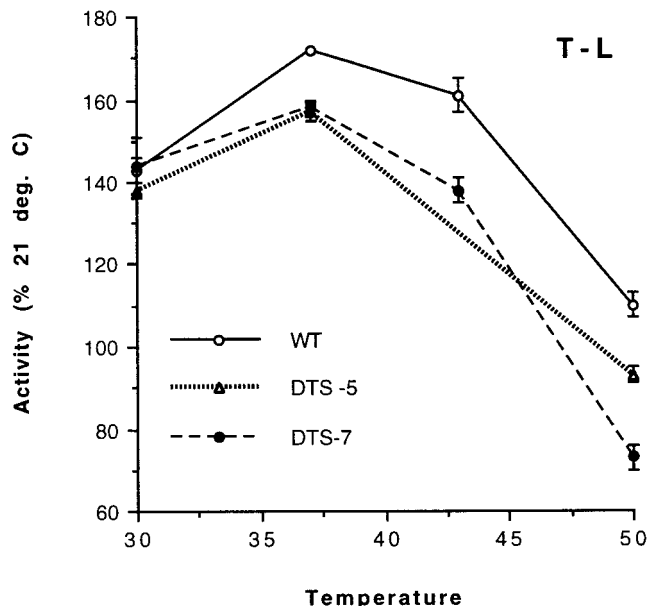


FIG. 6. Effects of elevated temperatures on trypsin-like activity of WT (+/+) and DTS/+ *Drosophila* proteasomes. Reaction mixtures contained 0.4 mM Cbz-GGR- β NA, 2 μ g proteasome, and 20 mM HEPES-NaOH (pH 8). Mixtures were preincubated for 1 h at 21, 30, 37, 43, or 50°C before addition of substrate. Reactions were incubated for 1 h at the preincubation temperature. DTS/+ proteasomes were less stable at higher temperatures than WT. Activities are expressed as percentages of control reactions at room temperature. Data for temperatures 30 and 37°C were collected in an assay separate from that containing the data for 43 and 50°C, but using the same enzyme preparations. During the assay inclusive of temperatures 30 and 37°C, the catalytic rates for WT, DTS5, and DTS7 reactions at room temperature were 1.15, 1.11, and 1.60 μ mol/mg MCP/h, respectively. In the same order, the room temperature catalytic rates for the assay inclusive of temperatures 43 and 50°C were 1.45, 1.83, and 1.98 μ mol/mg MCP/h, respectively.

cells (1). Fourteen genes encoding each of the seven alpha-type and seven beta-type subunits have been isolated as genomic and/or cDNA clones [(10, 11, 29–33), J. M. Belote and K. A. Smyth, unpublished observations]. Three additional genes, encoding two isoforms of the α 4/Pros28.1 subunit and one isoform of the α 3/Pros29 subunit, have also been identified [(34), J. Ma and J. M. Belote, in preparation]. All three of these isoforms are expressed exclusively in the male germline, during the mid- to late stages of spermatogenesis, and so they would likely have a minor impact on subunit diversity in complexes isolated from whole flies. Two isoforms each of mammalian C6-I, C2, and LMP7 are generated by alternative splicing (35–38), but nothing comparable has yet been described in *Drosophila*.

Changes in proteasome subunit composition occur during *Drosophila* development (12). Many of these changes result from posttranslational modifications, such as phosphorylation and proteolytic processing. Five of the seven alpha-type subunits in various species (zeta, C2, C3, C8, and C9) are phosphorylated *in vivo* (39–44). Only one (C5) of the seven beta subunits

in the rat liver complex has been isolated in a phosphorylated form (41). In *Drosophila*, putative phosphorylation sites occur in Pros35/C2 (30) and Pros28.1/C6-I (31), and several 28-kDa proteins separated by two-dimensional PAGE are phosphorylated *in vivo* (12). The presence of C2 species with different masses in proteasomes from *Drosophila*, hawkmoth, and lobster suggests that proteolytic processing of C2 is a common, and perhaps essential, feature [Fig. 1 (14)]. The functional significance is not known. Autocatalytic removal of N-terminal sequences of five beta subunits occurs during proteasome assembly, which, in the three catalytic subunits, exposes threonine residues that are essential for activity (45). N-terminal cleavage of C2 and C3 occurs in mammalian cells, but it is not associated with assembly (40, 46). In rat, processed C3 accumulates in the nucleus and may play a role in regulation of cell growth (40).

The most dramatic difference between WT and +/-DTS proteasomes is the appearance of a new subunit (#16) in DTS/+ complexes. This subunit was more abundant in DTS7/+ complexes than in DTS5/+ complexes (Figs. 1 and 2). Unfortunately, none of the subunit-specific antibodies currently available recognized the protein. Its small mass and lack of reactivity with an anti-alpha antibody (MCP222; see Figs. 1H and 1L) suggest that subunit #16 is the product of a beta gene. Not all beta genes have been sequenced in *Drosophila*, but of those that have been characterized, both β 3/C10-II and β 6/C5 are candidates, based on their estimated masses and pI values (Table I). β 2/Z is an unlikely candidate, since it has a higher estimated mass and alkaline pI. Moreover, the DTS7 allele of this subunit substitutes an arginine for a glycine, thus shifting the pI of the mutant polypeptide to a more positive pI. Alternatively, it may be a nonproteasomal protein associated with the DTS/+ complexes but not the WT complex. Although unlikely, this possibility cannot be eliminated until its identity is established by peptide sequencing.

The dominant negative effect of the DTS mutants suggests that DTS alleles encode poison subunits, which are incorporated into complexes and disrupt one or more functions essential for normal development (10, 11). A hypothetical model is presented in Fig. 7. Assembly is a multistep process in which seven different alpha and seven different beta subunits associate to form a two-ringed half-complex, two half-complexes dimerize via interaction at the beta rings, and proteolytic processing removes beta subunit N-terminal sequences to activate the complex (1, 45). Assuming equal expression of WT and DTS alleles, three types of complexes are present in +/-DTS heterozygotes in approximately a 1:2:1 ratio: +/+, DTS/+, and DTS/DTS. Since yields from +/+, DTS5/+, and DTS7/+ flies were similar, DTS subunits are assembled into proteasomes that are chromatographically indistinguishable from

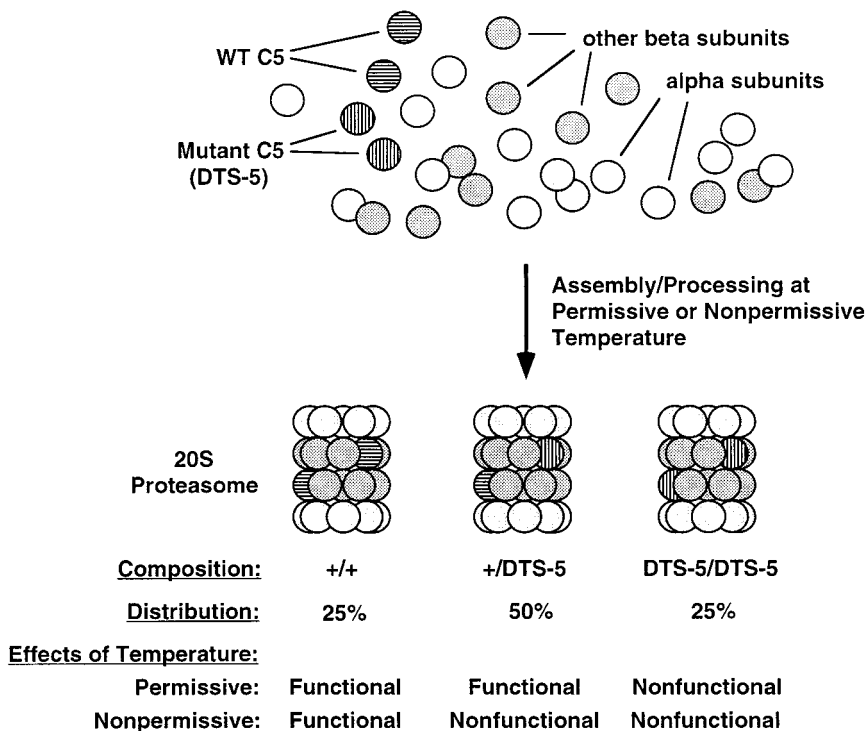


FIG. 7. Hypothetical model summarizing the effects of *DTS* alleles on proteasome structure and function. Genetic data support the hypothesis that DTS proteins act as “poison subunits” that disrupt proteasome function when incorporated into the particle. The biochemical data presented here suggest that DTS subunits affect assembly and/or processing of the 20S complex. For example, *DTS5* encodes a mutant form of the C5 beta subunit. Since there are two C5 subunits in each complex, three compositions are possible in *DTS5/+* heterozygotes: +/+, *DTS5/+*, and *DTS5/DTS5*. Regardless of temperature, +/+ complexes retain normal functions, whereas *DTS5/DTS5* complexes are nonfunctional. For *DTS5/+* complexes, assembly and processing produce particles that retain function at permissive temperature, but produce nonfunctional particles at nonpermissive temperature. The consequence is that the percentage of functional proteasome (25%) at nonpermissive temperature is not sufficient to sustain cell proliferation and differentiation at metamorphosis. In flies with an extra copy of the WT allele (*DTS5/++*), the percentage of +/+ complexes increases to about 44% (four-ninths), which apparently provides enough functional proteasomes for normal development at elevated temperature (10). This same model applies for the *DTS7* mutant subunit.

WT complexes. The model assumes that DTS subunits are assembled into complexes at all temperatures, since DTS-containing proteasomes are stable at elevated temperatures (Fig. 6). The dominant temperature-dependent phenotype is determined by the heterodimers containing one WT subunit and one DTS subunit, which constitute one-half of the population. Deleterious effects caused by the single DTS subunit are tolerated at permissive temperature, whereas they are not tolerated at nonpermissive temperature and result in a nonfunctional complex (Fig. 7).

Structural analysis of yeast and human proteasomes shows that $\beta 2/Z$ and $\beta 6/C5$ subunits interact across the beta rings (4, 7). Assuming the subunit topologies and structures of *Drosophila* are similar to those of yeast, the $\beta 2/Z$ (*DTS7*) subunit in one beta ring is positioned adjacent to the $\beta 6/C5$ (*DTS5*) subunit in the other beta ring. Both mutations are located in domains that mediate interactions between the two subunits (11). The missense mutation in *DTS7* (G170R) is located in a highly conserved loop sequence between α -helix 4 and β -sheet 9 of the $\beta 2/Z$ subunit (11); a magnesium ion

links this region (loop 162–167) to Asp 193 in $\beta 6$ in the yeast complex (4). The mutation in *DTS5* (T18I) is also positioned in a highly conserved loop sequence between β -sheets 2 and 3 (10); this region interfaces with $\beta 2'/Z$ (11). Thus, it is likely that DTS subunits incorporated into the two-ringed intermediates perturb dimerization, which results in an altered 20S complex. The two-dimensional PAGE analysis suggests that the altered complex is not posttranslationally modified in the same way as WT proteasomes. Since +/DTS heterodimers cannot be separated from DTS/DTS homodimers, it is not known whether differences in subunit composition result from disruption of posttranslational modification of homodimers (*DTS/DTS* complexes), heterodimers (+/DTS complexes), or both. Proteolytic processing of catalytic beta subunits is not affected, since proteasomes from +/+ and *DTS/+* flies have similar peptidase activities (Table III; Figs. 4–6).

The developmental phenotype of +/DTS heterozygotes may be due to a reduced amount of functional complex at nonpermissive temperature or to nonfunctional complexes interfering with cellular processes by

competing with functional proteasomes. Genetic studies cannot distinguish between these alternatives, since both are subject to stoichiometric expression of WT and DTS alleles. The proportion of functional proteasome required to sustain normal development is between 25 and 44%. The lower limit is determined from *DTS*/+ heterozygotes raised at nonpermissive temperature, conditions under which only the WT homodimers (25% of the population) would be functional. The upper limit is determined from heterozygotes with two copies of the WT allele (*DTS*/++); these flies develop normally at elevated temperature (10). Assuming equal expression and incorporation of WT and mutant subunits, the fraction of WT homodimers is four-ninths (about 44%) of the population at nonpermissive temperature.

Which functions are disrupted by the DTS subunits are not known. One obvious function, catalytic activity, is not greatly altered in DTS mutants. The five peptidase activities (T-L, nChT-L, PGPH, aChT-L, and BrAAP; SNAAP activity was not measured) are similar between proteasomes from +/+, *DTS5*/+, and *DTS7*/+ flies (Table III; Figs. 4–6). In other words, DTS/+ proteasomes retain catalytic function despite alterations in subunit composition. Perhaps this is not surprising since neither mutation is at a position essential for catalytic activity (10, 11). A second possibility is that mutant proteasomes are not directed to appropriate intracellular locations at specific times during development. Phosphorylation, for example, controls import of proteasomes into the nucleus (1) and developmental changes in intracellular distribution have been observed in *Drosophila* (13, 30), hawkmoth (28, 47), sea urchin (48), ascidian (49), and amphibian (50). A third possibility is that DTS subunits, either directly or indirectly, alter the binding of activators and inhibitors that regulate proteasome activities inside the cell. There are no data on the effects of posttranslational modifications or conformational changes on the binding of regulators. The mammalian C2 subunit contains a region that mediates binding of PA28 (51). The numerous C2 species in the *Drosophila* complex suggests that this subunit undergoes extensive posttranslational modifications, some of which may affect interactions with PA28 and other regulators.

In summary, these data show that incorporation of DTS subunits results in qualitative and quantitative changes in proteasome subunit composition at permissive temperature. These changes, however, have little if any effect on proteolytic activities and temperature stability of the complex. DTS subunits apparently act as poison subunits that disrupt proteasome functions necessary for normal development. According to our model, incorporation of a single DTS subunit is tolerated at permissive temperature, but not at nonpermissive temperature. Complexes containing two DTS subunits are nonfunctional at any temperature. The data

suggest that for DTS/+ complexes at nonpermissive temperature or for DTS/DTS complexes at permissive temperature assembly and/or processing is disrupted to such an extent that proteasome functions are compromised.

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