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# Control of *Drosophila* deoxyuridine triphosphatase

## Existence of a developmentally expressed protein inhibitor

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An activity that inhibits deoxyuridine triphosphatase (dUTPase) has been partially purified from *Drosophila melanogaster*. The inhibitor has a sedimentation coefficient of 4.1 S and a subunit molecular mass of 61 kDa. Its expression is limited to early stages of development, similar to the pattern previously found for dUTPase. The inhibitor is unusually stable to heating and is insensitive to DNAase and RNAase treatment. On the other hand, inhibition is sensitive to digestion with proteinase K, indicating that a protein is required for activity. These results suggest that at least one form of regulation is exerted on *Drosophila* dUTPase that could allow a greater opportunity for the incorporation of uracil into DNA.

## INTRODUCTION

Among the organisms studied, *Drosophila melanogaster* represents the only one to date that lacks an easily detectable uracil:DNA glycosylase for the removal of potential mutagenic sites in DNA caused by the deamination of cytosine to uracil [1–3]. On the other hand, a uracil-specific nuclease is produced, but only in late larval development [1]. In view of the transient expression of this nuclease, it was assumed that it most likely was not involved in DNA repair, but instead was part of some cellular design creating periodic breaks in DNA in the vicinity of dUMP residues that resulted in the DNA degradation observed in third-instar larvae (D. A. Green & W. A. Deutsch, unpublished work). As a consequence of these observations, investigations concerning the metabolism of uracil-containing compounds in *Drosophila* were undertaken. A strategic site for possible changes in uracil metabolism is at the step catalysed by deoxyuridine triphosphatase (dUTPase), which converts dUTP into dUMP and inorganic pyrophosphate *en route* to the formation of deoxythymidine triphosphate. The dUTPase enzyme has been purified from *Drosophila* embryos [4]; it is undetectable in other developmental states [4], with the possible exception of first-instar larvae, where variable levels of activity have been detected. It was anticipated that this decline in dUTPase activity was the result of its control at the level of transcription and/or through the action of an inhibitor. To test the latter possibility, we originally conducted complementation studies between the various developmental stages and purified embryonic dUTPase. While extracts from most of *Drosophila*'s predominant developmental stages had no effect on embryonic dUTPase, the addition of first-instar larval extracts reduced the activity of purified dUTPase. Preliminary studies indicated that the inhibitor present in crude extracts was unusually stable to heating [5]. On the other hand, inhibition could be inactivated by proteinase K treatment of first-instar larval extracts, suggesting that inhibition of dUTPase was most likely associated with a protein. In the present report we describe the partial purification and characterization of this inhibitor, which is present in early stages of *Drosophila* development.

## EXPERIMENTAL

### Materials

[5-$^3$H]dUTP (20 Ci/mmol) was from Amersham International.

### Purification of dUTPase

*Drosophila* dUTPase was purified as previously described [4]. Unless otherwise stated, the most highly purified forms of embryonic dUTPase were used, namely Fraction IV (phosphocellulose fraction) or Fraction V [50%-satd.-$(NH_4)_2SO_4$ fractionation of Fraction IV in which dUTPase was dissolved in 10 mM-Tris/HCl, pH 7.5].

### Collection of individual developmental stages

*Drosophila* embryos were washed with 70% ethanol and transferred to sterile dead yeast media [1]. Individual developmental stages were collected and washed as described previously [1].

### Assay of dUTPase and inhibitor activity

To monitor dUTPase activity, an assay using a DE81 Filter, which retains dUTP but releases dUMP [6], was used as previously described [4]. One unit of activity represents the production of 1 pmol of dUMP/min. One unit of inhibitor activity is defined as the amount that inhibits one unit of dUTPase activity.

### dUTPase complementation studies

*Drosophila melanogaster* larvae were collected and then stored in liquid $N_2$. Subsequently, first-instar (0.28 g), second-instar (0.06 g), and third-instar (0.36 g) larvae were thawed and homogenized in a glass micro-

Abbreviation used: dUTPase, deoxyuridine triphosphatase (dUTP nucleotidohydrolase, EC 3.6.1.23).

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homogenizer in 0.5–1.0 ml of larval homogenizing buffer (50 mM-Tris/HCl, pH 7.5/5 mM-dithiothreitol), centrifuged at 12000 *g* for 20 min, and filtered once through a Nitex screen.

### Affinity chromatography

*Drosophila* embryonic dUTPase (Fraction V) was coupled to epoxy-activated Sepharose 6B (Sigma) essentially according to the instructions of the manufacturer. Lyophilized powder (3 g) was suspended in and rapidly washed with sterile water. Following the removal of fluid on a sintered-glass funnel, the cake was mixed with 6 ml of purified embryonic dUTPase (0.48 mg) in 0.1 M-$NaHCO_3$ (pH 8.0). Following gentle shaking at 33 °C for 16 h, the suspension was washed with sterile water, followed by 0.1 M-$NaHCO_3$, pH 8.0/0.5 M-NaCl, and then 0.1 M-magnesium acetate, pH 4.0/0.5 M-NaCl. The suspension was blocked by 1 M-glycine at room temperature overnight. The binding of the embryonic dUTPase, as measured by the protein concentration of the glycine wash, had a 36% coupling efficiency.

First-instar larval extracts were applied to the affinity column (5 ml) described above that had been equilibrated with 50 mM-Tris/HCl, pH 7.0/0.2 mM-dithiothreitol (TD buffer). The column was washed with 3–5 ml of TD buffer, 3–5 ml of buffer containing 1 M-KCl, and 5 ml of 50 mM-Tris/HCl, pH 9.0/2 mM-dithiothreitol. Fractions collected contained 3–5 ml. dUTPase inhibitory activity appeared in the final washing. The fractions were usually concentrated to 100 $\mu$l portions in 10 mM-Tris/HCl, pH 7.0, by centrifugation in Amicon concentrators at 5500 rev./min in a Beckman JA-20 rotor.

### SDS/polyacrylamide-gel electrophoresis

Protein was reduced and denatured by heating at 70 °C for 4 min in sample (loading) buffer (60 mM-Tris/HCl, pH 6.5/1% SDS/5 mM-dithiothreitol) and loaded on to two separate lanes of a one-dimensional SDS/10% polyacrylamide gel with a 3.6% stacking gel. Electrophoresis was at constant voltage (150 V) for 4 h. One lane of the gel was stained with silver nitrate; the other was cut into 2 mm slices and then incubated in 0.5 ml of 20 mM-Tris/HCl (pH 7.5)/100 mM-NaCl/2 mM-dithiothreitol/1 mg of bovine serum albumin/ml while shaking at 4 °C for 16 h. Each slice was then assayed for inhibition of dUTPase.

### Sephadex G-100 gel filtration

To determine the molecular mass of the inhibitor, a 1.5 cm-diam. column of Sephadex G-100 packed to a height of 25 cm and equilibrated with 25 mM-Tris/HCl (pH 7.5)/1 mM-EDTA was precalibrated independently with Dextran Blue ($V_0$), bovine serum albumin (66000), carbonic anhydrase (28000), cytochrome *c* (12300), and Phenol Red ($V_c$). Fraction I (20.6 $\mu$g) was loaded on to the column and washed with equilibration buffer at 0.2 ml/min. Fractions (0.5 ml) were collected and concentrated by centrifugation in Amicon concentrators, and then monitored for inhibitory activity.

### Other methods

Protein was assayed by the Bradford technique using the Bio-Rad Protein Kit and the colorimetric micromethod of Boratynski [7]. The pH of all buffers was routinely measured at 50 mM and room temperature.

**Table 1. Embryonic dUTPase activity combined with extracts of the predominant stages of larval development**

Reaction mixtures (0.05 ml) contained 10 mM-potassium phosphate buffer (pH 9.1), 10 mM-dithiothreitol, 20 mM-[5-$^3$H]dUTP, individual larval extracts (first instar, 0.14 $\mu$g; second, 1.6 $\mu$g; third, 1.4 $\mu$g) and purified dUTPase (0.28 $\mu$g of protein).

| Experiment/addition | Embryonic dUTPase activity (pmol/min) |
|---|---|
| 1. None | 45.3 |
| First-instar larvae | 13.1 |
| 2. None | 46.7 |
| Second-instar larvae | 60.0 |
| 3. None | 47.6 |
| Third-instar larvae | 52.0 |

## RESULTS

### Detection of inhibitor activity in crude extracts

In order to determine if an effector molecule was responsible for the previously reported developmental decline in dUTPase activity [4], complementation studies were conducted between purified embryonic dUTPase and extracts from all other *Drosophila* developmental stages. While most had no effect on embryonic dUTPase, a decrease in activity was detected when the enzyme was combined with first-instar larval extracts (Table 1). Conditions under which enzyme and substrate were held constant, with increasing amounts of first-instar larval extracts added to reaction mixtures, resulted in the complete loss of embryonic dUTPase activity (results not shown). As expected, inhibition was relieved by increasing the amounts of dUTPase.

### Inhibitor purification

The binding of the inhibitor to dUTPase suggested a method of purifying the heat-stable protein. First-instar larvae were heated to inactivate heat-labile proteins, and the resulting extract (crude) was then applied to an affinity column containing embryonic dUTPase coupled to epoxy-activated Sepharose 6B. The inhibitor was subsequently eluted from the column with high-pH buffer. This resulted in a roughly 150-fold purification of the inhibitor (Table 2). It should be noted, however, that

**Table 2. Purification of inhibitor for dUTPase**

| Fraction | Volume (ml) | $10^{-3}$ × Total inhibitor units | Total protein (mg) | Specific activity ($10^{-3}$ × units/mg of protein) |
|---|---|---|---|---|
| Crude | 0.4 | 1.4 | 0.04 | 237 |
| Affinity column | 5.0 | 0.5 | < 0.01* | 34000 |

* The total protein for this fraction was 0.1 $\mu$g and was determined after concentration.

the homogenized first-instar larval extract was boiled and the resulting supernatant filtered through Nitex (crude fraction, Table 2), thus representing a substantial purification step prior to affinity chromatography. This was required since dUTPase activity was occasionally detected in first-instar larval extracts, the presence of which compromised the measurement for the inhibition of embryonic dUTPase; boiling the extract inactivated any residual dUTPase activity in crude extracts and therefore allowed for the reproducible detection of the inhibitor. This same strategy has been employed to see if dUTPase might be masking the presence of the inhibitor for other developmental stages in *Drosophila*. Small amounts of inhibitory activity were in fact detected in heat-denatured embryonic extracts. It therefore appears that the inhibitor is 'turned on' some time during embryonic development and persists into first-instar larval development; dUTPase expression probably spans the two stages as well, but is more narrowly confined to the transition between embryonic and larval development than the inhibitor.

### Molecular mass and subunit structure

Gel filtration of partially-purified (crude) extracts of first-instar larvae indicated a native molecular mass of roughly 58 kDa (Fig. 1). Sedimentation of the affinity-purified inhibitor in 5–30 % glycerol gave an $s_{20,w}$ value of 4.1 S and a molecular mass of approx. 60000, relative to marker proteins [8].

When concentrated samples of the affinity-purified inhibitor were electrophoresed through a 10% polyacrylamide gel (in the presence of SDS), activity could be recovered corresponding to a relative molecular mass of 60800 (Fig. 2). Although several silver-staining bands were present in this preparation, similar purifications have resulted in a single silver-staining spot, resolved by 2-dimensional SDS/polyacrylamide-gel electrophoresis, having a relative molecular mass of 54000 and an apparent pI between 5.2 and 4.5 (results not shown). Under these conditions, urea as opposed to SDS was used, and could have contributed to a lower molecular-mass estimate.

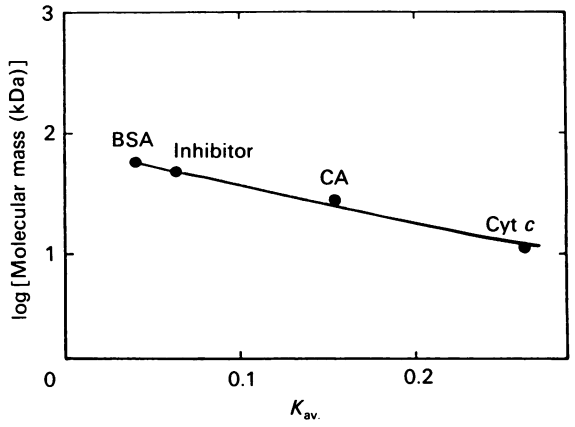


**Fig. 1. Gel-filtration of inhibitor**

Fraction I dUTPase inhibitor (20.6 $\mu$g) was applied to a Sephadex G-100 column equilibrated with 25 mM-Tris/HCl (pH 7.5)/1 mM-EDTA and precalibrated with bovine serum albumin (BSA; $M_r$ 66000), carbonic anhydrase (CA; 28000), and cytochrome *c* (Cyt.c; 12300).

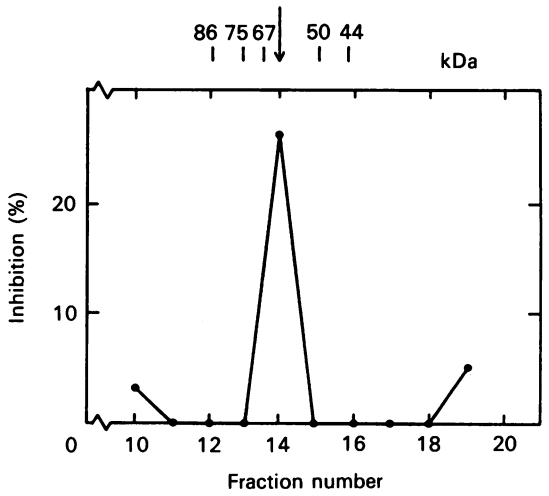


**Fig. 2. Polyacrylamide-gel electrophoresis of inhibitor**

Two lanes of a 10% polyacrylamide gel (in the presence of SDS) were loaded with less than 100 ng of the purified inhibitor and run as described in the Experimental section. Following electrophoresis, one lane of the gel was stained with silver nitrate, while the other lane was cut into 2 mm slices and assayed for inhibitory activity. The molecular masses (in kDa) corresponding to the marker proteins are shown.

**Table 3. Heat stability of the purified inhibitor**

Reaction mixtures (0.03 ml) contained 0.3 $\mu$g of purified embryonic dUTPase and 0.01 $\mu$g of purified inhibitor. Boiling was for 30 min.

| Addition | dUTPase activity (pmol/min) | Inhibition (%) |
|---|---|---|
| dUTPase | 8.4 | 0 |
| dUTPase + purified inhibitor | 3.5 | 58 |
| dUTPase + purified inhibitor (boiled) | 4.0 | 52 |

**Table 4. Effect of hydrolytic enzymes on the inhibitor of embryonic dUTPase**

Reaction mixtures (0.03 ml) contained 0.1 $\mu$g of purified inhibitor incubated with 0.3 $\mu$g of DNAase, RNAase, or proteinase-K for 10 min. Proteinase was inactivated by heating at 70 °C for 5 min. Purified embryonic dUTPase (0.25 $\mu$g) and reaction cocktail was then added and assays conducted as described in the Experimental section.

| Addition to incubations with dUTPase | dUTPase activity (pmol/min) | Inhibition (%) |
|---|---|---|
| None | 6.6 | 0 |
| Purified inhibitor | < 0.1 | 100 |
| DNAase + inhibitor | < 0.1 | 100 |
| RNAase + inhibitor | 0.6 | 90 |
| Proteinase K + inhibitor | 5.8 | 12 |

### Effect of heat and hydrolytic enzymes on inhibition

Previous results indicated that the inhibitor present in first-instar larvae was insensitive to heat treatment [5]. Since this result was obtained using crude extracts, similar tests were performed on the affinity-purified protein, in which the purified protein remained stable to boiling (Table 3). This suggested that the inhibitor may not be a protein. However, when the purified inhibitor was treated with proteinase K, activity was totally lost (Table 4). On the other hand, pancreatic DNAase or RNAase had no effect on inhibitor activity (Table 4).

## DISCUSSION

While this represents the first example of a eukarotic organism containing an inhibitor for dUTPase activity, there is a very similar type of prokaryotic protein found in PBS-infected *Bacillus subtilis*. Bacteriophage PBS is the only known organism that contains uracil instead of thymine in its DNA. This replacement of uracil for thymine is accomplished by the induction of a number of new proteins upon infection, some of which include an inhibitor of uracil:DNA glycosylase and dUTPase activity [9]. The inhibition of dUTPase allows for an increase in endogenous nucleotide pools of dUTP relative to dTTP, thus facilitating the incorporation of dUMP into DNA. The inhibition of uracil:DNA glycosylase is critical so that the incorporated uracil residues are not removed from the phage DNA. The latter activity has been purified [10] and recently cloned [11]. While it is not clear whether both inhibitor activities are one and the same, certain characteristics are shared by the bacterial inhibitor for uracil:DNA glycosylase and the inhibitor for *Drosophila* dUTPase. For example, both are extremely heat-stable, although the bacterial activity does become sensitive to heat inactivation in the most purified preparation [10]. Both are acidic proteins, especially the bacterial activity, which has a pI of 3.5 [10]. With regard to the bacterial activity, the highly acidic nature of the protein may have contributed to the acknowledged difficulties in obtaining an accurate molecular mass by SDS/polyacrylamide-gel electrophoresis [10]. On the other hand, the molecular mass of the *Drosophila* protein was obtained by three different means, all resulting in a value of approx. 60 kDa. Furthermore, while it might be suggested that the small size of the bacterial enzyme might confer heat stability, the same could not be true for the *Drosophila* protein.

A question that remains is whether the inhibitor acts stochiometrically, or catalytically by the remote possibility that it proteolytically inactivates dUTPase. Further studies, where substantially more protein is available, should clarify this concern. In this regard, questions of this nature still remain about the inhibitor found in PBS-infected *Bacillus subtilis*, although the molecular cloning of this gene may eventually provide some of the answers concerning the mode of action of this inhibitor. Likewise, this may be the most direct route in understanding the nature of dUTPase control in *Drosophila*.

Even though the precise role of the inhibitor is unknown, certain predictions seem warranted. For example, it seems reasonable that one outcome of dUTPase inhibition is altered intracellular dUTP pools, thereby increasing the likelihood of dUMP incorporation into *Drosophila* DNA.

Examples exist in which the incorporation of dUMP into DNA results in strand breakage [12,13] and shortened Okazaki fragments [14,15] as a product of excision repair. The same events, on a more moderate scale, could be predicted for *Drosophila* development, especially as it relates to DNA degradation associated with the cellular histolysation process in pupae. It will be interesting to learn in future studies if indeed the inhibition of dUTPase leads to an eventual target for the previously described uracil nuclease [1]; if so, this would imply a designed or programmed scheme leading to the eventual death of a cell through DNA degradation.

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