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Comparative Biochemistry and Physiology Part B 124 (1999) 423–427

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The effect of urea exposure on isoaspartyl content and protein L-isoaspartate methyltransferase activity in *Drosophila melanogaster*

Cynthia L. David [a,1], Valerie A. Pierce [b,2], Dana W. Aswad [a,*], Allen G. Gibbs [b,3]

[a] *Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, CA 92697-3900, USA*
[b] *Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, CA 92697-2525, USA*

Received 24 May 1999; received in revised form 12 August 1999; accepted 16 August 1999

Abstract

Urea is a protein unfolding agent that can accumulate to locally high concentrations in tissues of many organisms. We used *Drosophila melanogaster* to test the hypothesis that urea loading would promote formation of isoaspartate (β-carboxyl-linked aspartate), a common form of protein damage that occurs most readily in unstructured polypeptides and flexible regions of folded proteins. Ten populations of flies were tested; five control populations of urea-sensitive flies and five previously selected urea-tolerant populations. We measured the effects of urea consumption on levels of both isoaspartate and protein L-isoaspartate methyltransferase (PIMT), an enzyme believed to function in the repair or removal of isoaspartyl proteins. For both sets of populations, urea feeding for 6 days increased isoaspartyl levels by approximately 60%, supporting the idea that disruption of protein secondary and tertiary structures can accelerate the formation of isoaspartate in vivo. Urea feeding tended to increase PIMT activity in both control and urea-tolerant populations. There were no significant differences in PIMT activities or isoaspartyl levels between the control and urea-tolerant flies raised on normal or urea food. The latter findings indicate that urea tolerance evolved in the selected populations without any significant change in PIMT expression or activity. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Adaptation; *Drosophila*; Evolution; Isoaspartate; Laboratory selection; Methyltransferase; Protein damage; Urea

1. Introduction

Urea is a cytotoxic compound, which nevertheless accumulates to high levels in mammalian kidneys, the tissues of elasmobranch fishes (rays and sharks), and in other biological systems [23]. Urea destabilizes proteins and nucleic acids, and is therefore commonly used as a denaturant in the laboratory. Organisms which accumulate urea generally also amass other compounds, such as trimethylamine oxide, which stabilize protein structure and counteract urea's harmful effects [23]. Intracellular urea concentrations in these animals can exceed 300 mM, levels that are toxic to most cells. Resistance to urea's toxic effects can be achieved in the laboratory by selectively breeding urea-tolerant individuals. Mueller and colleagues subjected populations of the fruit fly, *Drosophila melanogaster*, to increasing levels of dietary urea for 25 generations [10]. In the larval stages, these populations live in and consume media containing 300 mM urea, which causes nearly complete mortality in unselected *D. melanogaster*. We are interested in elucidating the biochemical bases responsible for the selected flies' ability to survive in this environment. Recent work by some of us has demonstrated that the selected flies contain less urea than the control flies [18]. It is unknown if the selected flies accomplish this by decreasing uptake of urea, increasing urea excretion, or by some other means.

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; ANOVA, analyses of variance; dpm/pmol, disintegrations min^{-1} $pmol^{-1}$; PIMT, protein L-isoaspartate methyltransferase; TCA, trichloroacetic acid.

* Corresponding author. Tel.: +1-949-824-6866; fax: +1-949-824-8551.

E-mail address: dwaswad@uci.edu (D.W. Aswad)

[1] Present address: Department of Pediatrics, University of Arizona, Tucson, AZ 85724, USA.

[2] Present address: Department of Biology, College of Staten Island/CUNY, Staten Island, NY 10314, USA.

[3] Present address: Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA.

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PII: S0305-0491(99)00135-2

Another way to ameliorate urea's toxic effects would be to repair the damage it causes. Larvae adapted to the presence of urea may have increased their resistance to protein damage, or increased their ability to repair damaged proteins. As proteins relax their tertiary structures, they become vulnerable to digestion by proteases, and to nonenzymatic chemical reactions such as oxidation of the sulfur atoms of methionine and cysteine and formation of isoaspartate. Isoaspartate (β-carboxyl-linked aspartate) is generated within labile sequences by the deamidation of asparagine or isomerization of aspartate. Most organisms, including fruit flies, have an enzyme, protein L-isoaspartate methyltransferase (PIMT) (EC 2.1.1.77), which specifically methylates the carboxyl moiety on L-isoaspartyl groups [9,12,15–17]. This enzyme is believed to function in protein repair, because the methyl ester reaction products are unstable and spontaneously break down, ultimately forming a mixture of aspartate- and isoaspartate-containing proteins in a 1:3 ratio ([2] and references therein). In vitro experiments have shown that recycling through this pathway converts most of the original isoaspartate to aspartate [8,14].

Because urea is a protein denaturant, we hypothesized that larvae fed urea-containing media would contain higher levels of isoaspartate than larvae fed normal media. They might also express PIMT at higher levels in order to repair these proteins, and populations selected for urea tolerance may have higher levels than unselected control populations. To distinguish between phenotypic responses to urea and evolved changes due to selection for urea-tolerant *D. melanogaster*, we compared selected and control populations reared on both urea-containing and normal food. Our results suggest that dietary urea caused increased protein damage, as indicated by isoaspartyl levels, and that PIMT activity was mildly increased under these conditions. However, isoaspartyl and PIMT levels did not differ between urea-selected and control populations, on either food treatment, indicating that selection for urea tolerance did not affect these parameters.

2. Materials and methods

2.1. Materials

Bovine γ-globulins were purchased from Sigma. Radiolabeled *S*-adenosyl-[*methyl*-^{3}H]L-methionine ([^{3}H]AdoMet) was purchased from New England Nuclear. Unlabeled AdoMet was purchased from Sigma and purified on carboxymethyl cellulose [4] before using it to dilute the [^{3}H]AdoMet to the desired specific activity. Recombinant rat PIMT was purified as described [6] and had a specific activity of 15 200 U/mg, where 1 U is defined as 1 pmol methyl transferred to bovine γ-globulins per min at 30°C.

2.2. Experimental populations and selection protocol

The flies were derived from five baseline ('B') populations originally founded by Michael R. Rose [20]. These flies have been reared since 1980 on banana medium (normal food), under a 14-day generation cycle, at 25°C, with constant illumination. A urea-selected (MX) population was derived from each B population by providing larvae with banana medium containing urea (urea food) [10]. Adults were maintained in population cages with normal food, so that only the larvae were exposed to urea. The initial urea concentration was 200 mM, which was gradually increased to 300 mM over the first 25 generations. At the time of our experiments, the populations had undergone greater than 100 generations of selection. Five control (UU) populations were also derived from the B flies. These were reared under conditions identical to the MX populations, except that the larvae developed in urea-free medium (normal food).

2.3. Experimental design and statistical analysis

To remove parental effects, the populations were raised under identical, nonselective conditions for one generation before the experiment was begun. The experimental generation consisted of four groups, each replicated fivefold: control populations reared on normal and 300 mM urea food, and urea-selected populations reared on normal and 300 mM urea food. Control populations reared on urea food develop through the larval stages, albeit more slowly than on normal food [10], but suffer nearly complete mortality during pupation [21]. All measurements were done on third-instar wandering larvae, the stage just prior to pupation. Two-way analyses of variance (ANOVA) on population means were used to examine the effects of selection treatment and food type and the interaction of these terms on the traits measured. Two statistical tests, Dixon's and Grubb's tests, were used to identify outliers [22]. All data are presented as means of populations $(n = 5) \pm 1$ S.D. All analyses were performed using Minitab version 10 or SYSTAT for Windows.

2.4. Extract preparation

Each extract was prepared from 40 larvae which had been washed with 400 mM sucrose after collection and stored at −70°C. The larvae were homogenized in 300 µl homogenization buffer (100 mM Na phosphate pH 6.8, 2 mM EDTA, 15 mM β-mercaptoethanol, 100 µM phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin) with 30 strokes in a 1.5-ml microfuge tube with a Kontes glass pestle. Homogenates were centrifuged at $15\,800 \times g$ for 10 min at 4°C. Supernatants were recovered and glycerol was added to a final concentration of 5%. Extracts were stored at −70°C.

2.5. PIMT assay

Extract (106 μg protein) was mixed with 20 μM [^{3}H]AdoMet (14 000 disintegrations min^{-1} $pmol^{-1}$ (dpm/pmol)), 500 μg γ-globulins in 10 mM HCl, 100 mM Na phosphate, 0.5 mM EDTA, pH 6.7 in 1.5-ml microfuge tubes. Final reaction volume was 50 μl. Blank reactions to measure endogenous methylation consisted of the same mixture without γ-globulins added. The reactions were incubated at 30°C for 15 min. Reactions were stopped with 0.7 ml of 7% trichloroacetic acid (TCA), vortexed, and placed on ice for 5 min. If the reaction did not include γ-globulins, 10 μl of a 25 mg/ml solution of γ-globulins in 10 mM HCl was added after the TCA. Tubes were centrifuged at $12\,000 \times g$ for 30 s, supernatants were aspirated and discarded, and the pellets were washed with 0.7 ml H_2O. Pellets were dissolved in 100 μl 2% MeOH, 0.2 N NaOH, and 80 μl was spotted onto accordion-pleated filter paper lodged in a shell vial top. The top was then placed over a shell vial containing 2.5 ml Liquiscint (National Diagnostics). Vials were heated at 40°C for 1 h to allow the hydrolyzed [^{3}H]CH_3OH to diffuse into the cocktail, then the filter papers were removed and radioactivity in the vials was measured by a liquid scintillation counter. The reported enzyme activity is the methylation measured in the presence of γ-globulins minus the methylation measured in the absence of γ-globulins.

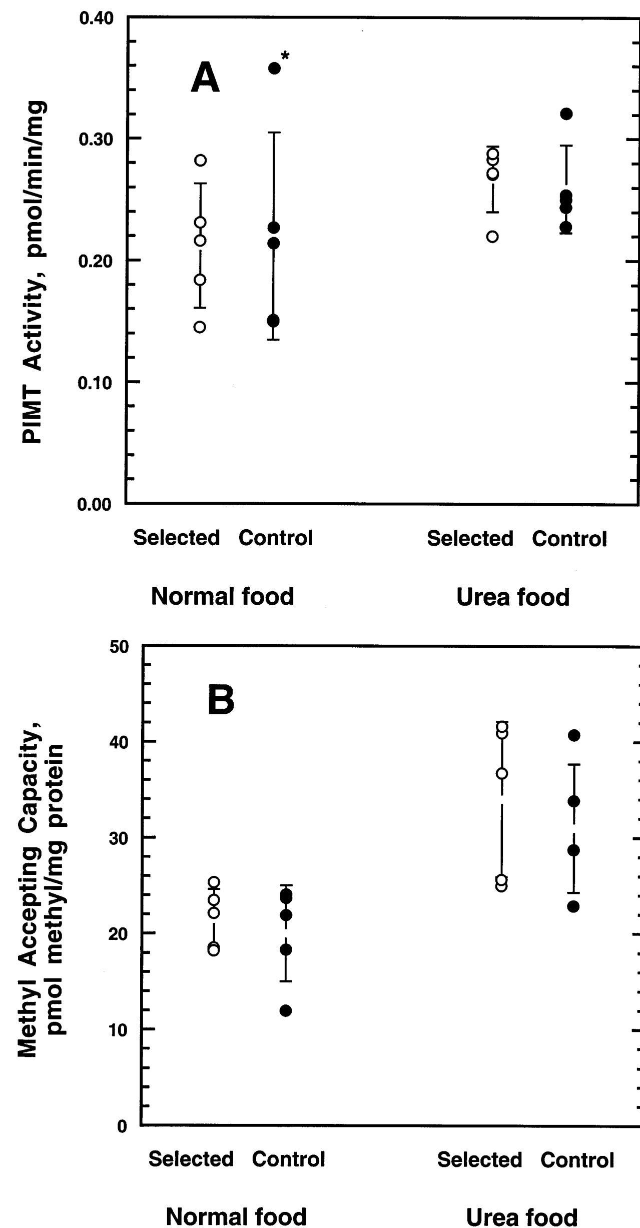

Fig. 1. (A) Variation in PIMT activity and (B) protein isoaspartyl content in selected and in control *D. melanogaster* populations. Open circles, flies selected for urea tolerance; closed circles, control flies. Enzyme activity and protein methyl accepting capacity were measured as described in Section 2. Each point is the mean of two measurements for a given population, error bars represent S.D. An asterisk indicates a data point that may be an outlier (see Section 3).

2.6. Methyl-accepting capacity

Reactions consisted of 66.5 μg extract proteins, 36 μM [^{3}H]AdoMet (6700 dpm/pmol), 1.1 μM PIMT, in 62 mM Na phosphate, 20 mM Na citrate, 2 mM EDTA, pH 6.2. Final reaction volume was 50 μl. The reactions were started by the addition of extract proteins. Microfuge tubes (1.5 ml) were incubated at 30°C for 15 min. Blank reactions contained homogenization buffer instead of extract protein. Reactions were stopped with 0.7 ml of 7% TCA, 250 μg γ-globulins were added, and the samples were processed as described above for the PIMT assay.

2.7. Other methods

Protein concentrations were measured using the method of Lowry et al. [13] after precipitation in a final concentration of 5% TCA. Urea concentrations in the larvae were determined spectrophotometrically as described previously [18].

3. Results

The effects of urea feeding on PIMT activity and isoaspartyl levels in extracts of *D. melanogaster* are shown in Fig. 1. PIMT activity was similar in the selected and control populations. Although there was an elevation in enzyme levels for both sets of flies when the flies were fed urea-containing food, the increase was of low statistical significance ($P < 0.07$). One control population on normal food had a PIMT activity 58% higher than that of the next highest population in the same treatment group (Fig. 1A). Both Dixon's and Grubb's tests rejected the hypothesis that this data

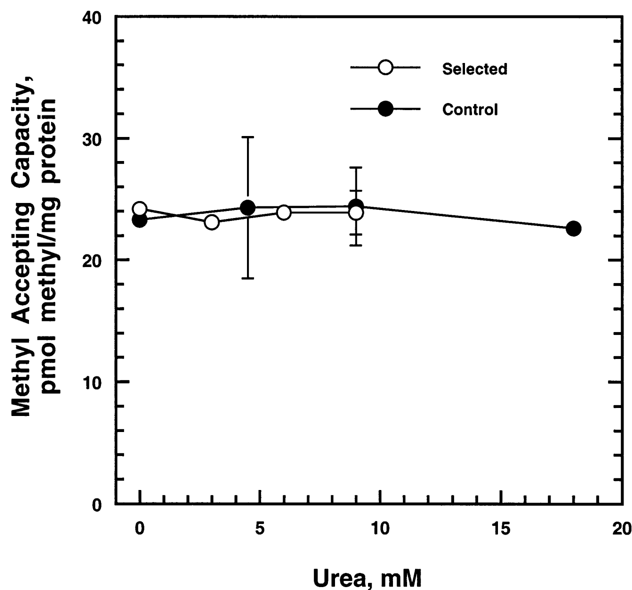

Fig. 2. Exogenous urea does not change the isoaspartyl content of *D. melanogaster* larval extracts. Open circles, flies selected for urea tolerance; closed circles, control flies. Pools were made from selected and control extracts prepared from larvae reared on normal food. Urea was added to the extracts, which were then assayed for methyl accepting capacity as described in Section 2. Each point is the mean of three measurements, error bars represent S.D. Where no error bars appear, the S.D. was less than 4% of the mean.

point was an outlier at the $P < 0.05$ level but accepted it at the $P < 0.10$ level. When an ANOVA on PIMT activity was performed without including this data point, PIMT activity was higher in both control and selected populations reared on urea food ($P = 0.003$). These analyses suggest that PIMT activity may be increased when the flies are fed urea food. However, because the effect was not selection-treatment dependent, we conclude that increased expression of PIMT is not one of the mechanisms used by the urea-tolerant flies to survive the urea exposure.

Protein isoaspartyl content, as measured by methyl-accepting capacity, was the same for the selected and control flies, $P = 0.414$ (Fig. 1B). However, when the flies were fed urea-containing food, the isoaspartyl content of extract proteins rose by 55–58%, and the increase was highly significant ($P < 0.001$).

We considered the possibility that the increased isoaspartyl levels in urea-fed larvae might be due simply to the presence of urea in the assay mixture, which could partially unfold proteins and expose more sites to methylation by PIMT. To test this possibility, we added 0–18 mM urea to extracts prepared from larvae reared on normal food. (Extracts from urea-fed larvae contained less than 10 mM urea [18].) Extracts were incubated with urea on ice for 1 h, then the methyl-accepting capacity was determined as described in Section 2. We detected no difference in the methyl-accepting capacity of these extracts, regardless of the concentration of urea used (Fig. 2). Therefore, we conclude that the increase seen in protein isoaspartyl levels between flies fed normal food and flies fed urea food was due to isoaspartate forming in the urea-fed flies in vivo, and not in the extracts in vitro.

4. Discussion

After greater than 100 generations of selection, the selected *D. melanogaster* populations are able to grow and develop in food containing 300 mM urea. The physiological mechanisms allowing resistance to urea are poorly understood. Larvae from the selected populations cannot exclude urea from their tissues, although they contain lower levels than the controls (79.1 vs 125.6 nmol urea/mg larvae) [18]. Urea is generally considered to be a membrane-permeant compound, although specific transport proteins are found in many tissues [11]. There is no evidence that the selected populations accumulate urea-counteracting solutes such as trimethylamine oxide [18]; thus, one would expect proteins to be destabilized in larvae fed urea. Increased protein flexibility results in higher rates of isoaspartate formation in vitro [1,3,5,7,19] and may contribute to the toxic effects of urea.

We found that urea exposure increased in vivo isoaspartate formation in *D. melanogaster*. Both control and selected larvae had greater than 50% higher isoaspartyl levels when reared on urea food compared to rearing on normal food. The level of isoaspartate in extracts of the selected flies was the same as that in the control flies, suggesting that selection for urea tolerance has not produced genetic adaptations that reduce this type of protein damage. We also observed a small increase in PIMT activity in larvae reared on urea food. Again, there was no difference in activity between the control and selected larvae, indicating that PIMT levels have not changed in response to urea selection. This absence of an evolved change may explain why we see no reduction in isoaspartyl levels in the selected larvae.

Several possible explanations exist for the lack of evolved differences in isoaspartyl and PIMT levels between the selected and control populations. First, isoaspartate accumulation may not contribute to the death of these larvae, and thus selection has not reduced its levels. Second, there may be insufficient genetic variation for PIMT activity, and thus selection would have no variation on which to act. Third, reducing isoaspartyl levels may indeed be beneficial to urea-fed larvae, but increased PIMT levels may have additional, detrimental effects on survival. Thus, counteracting selective forces may result in no net change in PIMT activities. Finally, the PIMT gene could be closely linked to a locus that is even more important for

urea tolerance. Strong selection on the linked gene may have restricted the evolution of increased PIMT activity. These hypotheses can be tested, by subjecting populations to stronger urea selection, by genetically manipulating PIMT levels, and by using different founding populations, which will contain a different suite of genetic variation.

Acknowledgements

This work was supported by NIH grant NS17269 to D.W. Aswad, NSF grant IBN-9317471 to A.G. Gibbs, and UCI Multi-Investigator Award to A.G. Gibbs, D.W. Aswad, and L.D. Mueller. We thank Jason Lee for assisting in extract preparation and Kasra Rowshan for protein determinations. We thank Dr Laurence Mueller for providing *D. melanogaster* and for comments on the manuscript.

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