Genetic and Hormonal Control of Cytosolic Malate Dehydrogenase Activity in *Drosophila Melanogaster*

R. FARKAŠ AND J. KNOPP

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Slovakia

Abstract. The activity of *Drosophila* cytosolic malate dehydrogenase (MDH; EC 1.1.1.40), a key enzyme in the biosynthesis of lipids, was found to be regulated by both the sesquiterpenoid juvenile hormone (JH), and the steroid hormone ecdysone. The responsiveness of MDH to JH largely depended on the developmental stage or endogenous titre of ecdysone. During the early through middle period of the last larval instar, when ecdysone levels are low, MDH responded to JH rapidly by increasing activity, while little or no response was measured in mature larvae (postfeeding stage) and fresh pupae when the endogenous pulse of ecdysone is high. Activity of MDH in ecd^1 and $su(f)^{ts67g}$, two ecdysone-deficient mutants of *Drosophila*, was increased when compared to wild type controls, and was also sensitive to administration of JH. The differences in MDH activity between ecd^1 and $su(f)^{ts67g}$ were negligible indicating a substantial role of ecdysone in the enzyme regulation and minimal or no effect of their genetic backgrounds. Accordingly, another *Drosophila* mutant, ap^4 which is naturally deficient in JH production, displayed significantly reduced activity of MDH in heterozygotic combination, and almost undetectable MDH activity in null homozygote adults. The ap^4 phenotype was more strongly manifested in the adult stage than in larvae which showed 7-times lower requirements for JH titre. In addition, high/low sucrose diet fed to wild types or mutants affected the activity of larval MDH, but the enzyme remained sensitive to administration of JH. These results corroborate those described for mammals and provide the first evidence that *Drosophila* MDH might be under differential hormonal and nutritional control. These data will serve as a basis for further molecular characterization of the *Drosophila* MDH gene and its regulation.

Correspondence to: Robert Farkaš, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Slovakia. Tel.: (++4217) 373800 Fax: (++4217) 374247 E-mail: Farkas@uee.savba.sk

Introduction

Cytosolic malate dehydrogenase or malic enzyme (MDH; EC1.1.1.40) has a central role in the biosynthesis of lipids by catalyzing the NADP-dependent oxidative decarboxylation of malate to pyruvate plus carbon dioxide, and generating NADPH. In mammals and other vertebrates, this enzyme is under both nutritional and hormonal control. Vertebrate MDH is a target for action of several hormones: catecholamines, insulin, thyroid hormone and retinoic acid (Ballard and Hanson 1967; Geer et al. 1976; Towle et al. 1980; Dozin et al. 1985; Morioka et al. 1989; Hernandez et al. 1993). Nutritional control for MDH activity was also demonstrated in the fruitfly, *Drosophila melanogaster*, a widely used molecular and genetic model organism. It was shown that the activity of MDH is low at the beginning of postembryonic development and gradually increases during the larval life culminating in the first half of the last larval instar; it is lowered during the pupal stage, and then increases again with age in adults (Redkin 1970; Geer et al. 1978b; Chernik et al. 1982; Kaplin and Korochkin 1987). The course of MDH activity during the larval development has a fixed pattern but it may be modulated by dietary factors. A high saccharide diet results in increase of MDH activity in feeding larvae, while a low saccharide/high lipid diet causes MDH activity to drop to almost undetectable levels (Geer et al. 1976, 1978a, 1980; Geer and Perille 1977). Recently we have found that the activity of the *Drosophila* MDH is upregulated by sesquiterpenoid juvenile hormone (JH) produced by corpora allata, an endocrine gland of insects located near the brain, and that the response of MDH to JH or JH analogues (JHA) is modulated by an insect steroid hormone, ecdysone (Farkaš and Knopp 1997). Briefly, this response covers a period of more than 20 h, it is continuous and has 2 distinct phases. The first phase is characterized by a rapid increase of MDH activity visible 1 h after JH treatment and it lasts for 2–3 h. This phase is independent of RNA and protein synthesis. During the second phase, increase of MDH activity continues for 10–16 h and may reach 3–4 fold higher values, than in controls; the second phase was sensitive to actinomycin D, α-amanitin, cycloheximide and puromycin, suggesting that transcription and/or translation were involved in the later phases of MDH activity increase (Farkaš and Knopp, unpublished observations). Measuring MDH during almost complete postembryonic life span, from 2nd larval instar through adult stage, revealed two important and peculiar aspects of developmental regulation of the enzyme activity: [i] during the larval development, MDH reached its maximum values when the endogenous titre of ecdysone is known to be low, and *vice versa*, its minimum values at high levels of endogenous ecdysone; [ii] in adults, markedly in females, MDH activity has coincided with periods of moderate to slightly increased ecdysone levels and also with apparent production of JH. Here, we present additional and supportive evidence about JH action on *Drosophila* MDH by using gene allele of *apterous*^{4} (*ap*^{4}), a JH-deficient

mutant (Altaratz et al. 1991; Bourgouin et al. 1992; Cohen et al. 1992), and two ecdysone deficient mutants, $l(3)ecd^{1ts}$(= *ecdysoneless*^{1}) commonly referred to as ecd^1 (Garen et al. 1977), and *suppressor of forked*^{ts67g} ($su(f)^{ts67g}$) (Snyder and Smith 1976; Klose et al. 1980; Hansson and Lambertson 1983).

Materials and Methods

Rearing, staging and hormonal treatments of flies

All experiments were performed on a wild-type strain *Oregon R*, two temperature sensitive conditional mutants, ecd^1 and $su(f)^{ts67g}$, and on ap^4 balanced over $SM5$ or CyO chromosome of *Drosophila melanogaster*, obtained from Indiana University Drosophila Stock Center, Bloomington, USA, and from Umea Drosophila Centre, Umea University, Sweden. Flies were cultured in 50 ml bottles at 23 °C on ~ 10 ml of agar-yeast-cornmeal-sucrose medium (Ransom 1982) with the addition of nipagin to inhibit mold growth. Regular diet contained 0.5% sucrose while high-sucrose medium contained 5% sucrose which is known to increase larval MDH activity; higher concentrations of sucrose do not have more stimulatory effects (Geer and Perille 1977; Geer et al. 1978a). 20-Hydroxyecdysone (Rotho Pharmaceutical Co., Osaka, Japan) was applied to the food in the way that one hundred microliters containing 1 μg of the hormone at desired concentration in 5% ethanol were mixed with an equal volume of Fleishman's yeasts until a dough-like consistency was obtained; excess ethanol was evaporated by placing the mixture into 29–30 °C for 0.5 h, and the mix applied on the top of the food in the vial with the larvae.

Juvenile hormone III (Sigma Chemical Co., St Louis, USA) and juvenile hormone analogue, methoprene (Zoecon Corporation, Palo Alto, USA) were applied in 0.5 μl of acetone topically with a Hamilton microliter syringe type 7000 on the top of larvae, puparia or adults, and at the dose 0.01 μg of JH III/animal, and 0.001 μg of methoprene/animal, respectively.

Preparation of Drosophila homogenates and determination of malate dehydrogenase activity

MDH activity was measured principally by the procedures of Ballard and Hanson (1967) and Freedland et al. (1968) as modified by Knopp et al. (1992). The crude extract was centrifuged at 12,000 rpm for 15 min at 4 °C to remove cellular debris, and the resulting supernatant was spun in a Ti65 rotor in a Beckman preparative ultracentrifuge L8–70 for 1 h at 40,000 rpm. The 100,000 × *g* supernatant was used for spectrophotometric determination of MDH activity at 340 nm on Zeiss-Opton 3C UV/VIS spectrophotometer as assessment of NADP reduction after addition of L-malate. The protein contents of the cytosolic fraction of *Drosophila* homogenates was quantitated by using dye-binding method of Bradford (1976). Final activity of

MDH enzyme (in ncat which represent nmol of formed NADPH per second) was calculated against a calibration curve made with each experiment. All values of enzyme activity are means of 4 determinations, and the standard deviations are provided to indicate the degree of variance. Student's t-test was applied to determine the significance of the findings. Where appropriate, enzymatic activity was then related to known values of ecdysteroid titre during *Drosophila* development as reported by Ashburner (1989) and Riddiford (1993), from papers of Borst et al. (1974), De Reggi et al. (1975), Hodgetts et al. (1977), Berreur et al. (1979, 1984) and summarized in Richards (1981).

Results

During normal larval development towards the end of the last instar the activity of MDH is progressively declining to low levels (Fig. 1*A*). Higher levels of MDH activity were found in larvae fed on high sucrose diet (Fig. 1*B*). If JH was applied to mid or feeding late 3rd instar larvae there was a significant rise in MDH activity (Fig. 2*A*) which was even more remarkable in high sucrose-fed larvae (Fig. 2*B*). As we reported elsewhere, if ecd^1 and $su(f)^{ts67g}$ mutants were used and exposed to and kept at restrictive temperature (29°C) at the time of moult from the 2nd to the last instar, MDH activity of identically aged larvae stayed at high levels comparable to the first half of the 3rd instar; the differences in MDH activity between the two mutants were negligible, and thus measured values are plotted as single curve in a graph. On the contrary, ecd^1 or $su(f)^{ts67g}$ larvae artificially fed on 20-hydroxyecdysone, starting two or more days after a shift to 29°C, had greatly reduced activity of MDH, and they showed signs of abortive pupariation (Farkaš and Knopp 1997). Also, application of JH or JHA to ecd^1 and $su(f)^{ts67g}$ mutant larvae shifted to restrictive temperature have further increased MDH activity. When the same experiment was performed with larvae fed on a high sucrose diet known to stimulate lipogenesis, MDH activity in ecd^1 or $su(f)^{ts67g}$ animals increased by another 20–25% in comparison to normal fed mutant larvae (Fig. 3*A*). In addition, JH stimulated another and reproducible, albeit small, increase in activity of MDH in high sucrose-fed ecd^1 and $su(f)^{pts67g}$ mutants (Fig. 3*B*). It should be noted that these effects of JH on MDH seemed to disappear about 50–60 h after the application when the enzyme activity had returned back to values similar to JH-untreated larvae. However, these values were still higher than in wild types or in ecd^1 and $su(f)^{ts67g}$ mutants not exposed to restrictive conditions.

An interesting situation was observed in ap^4 mutants. Freshly eclosed adult males and females of this homozygote have undetectable levels of MDH activity (Fig. 4*A*), part of the complex phenotype, which is rescuable by exogenous administration of JH or JHA to freshly emerged flies or even to exarate (pharate) adults; the sooner JH or JHA was applied on exarate animals, the higher MDH ac-

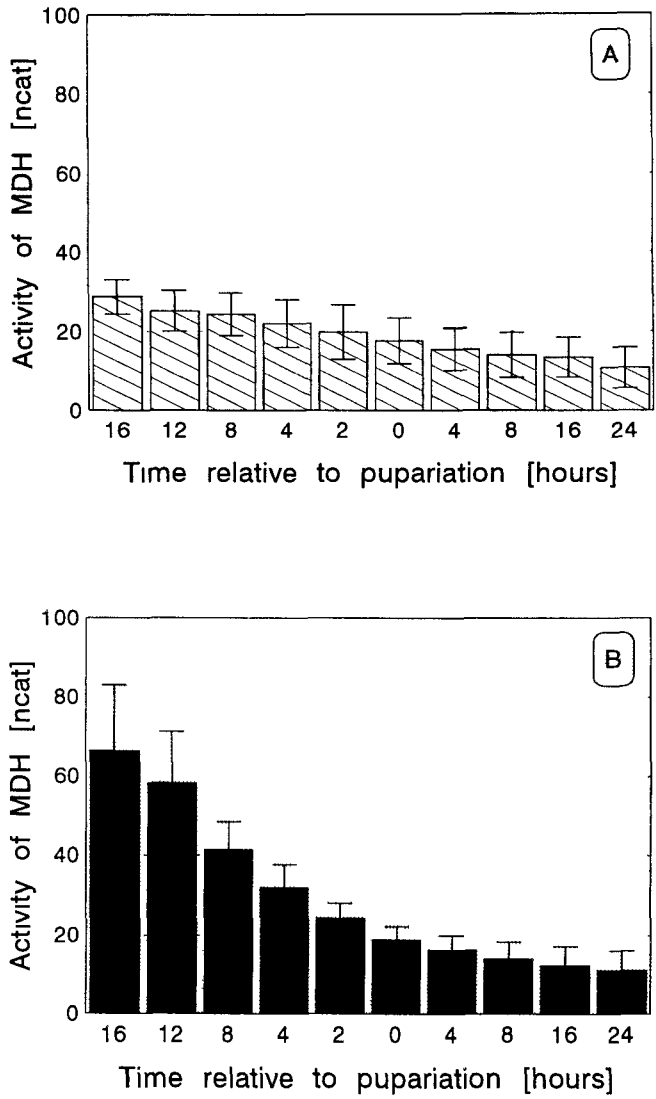

Figure 1. (*A*) The course of MDH activity in wild type (control or sham-treated) larvae of the last instar This course is characterized by a gradual decline of the enzyme activity during the final hours of larval development (*B*) Increase in MDH activity in wild type larvae fed high sucrose diet Note that the increased activity is mainly associated with the feeding period of the last larval instar, and with the approaching pupariation it is downregulated to levels comparable to animals fed normal diet

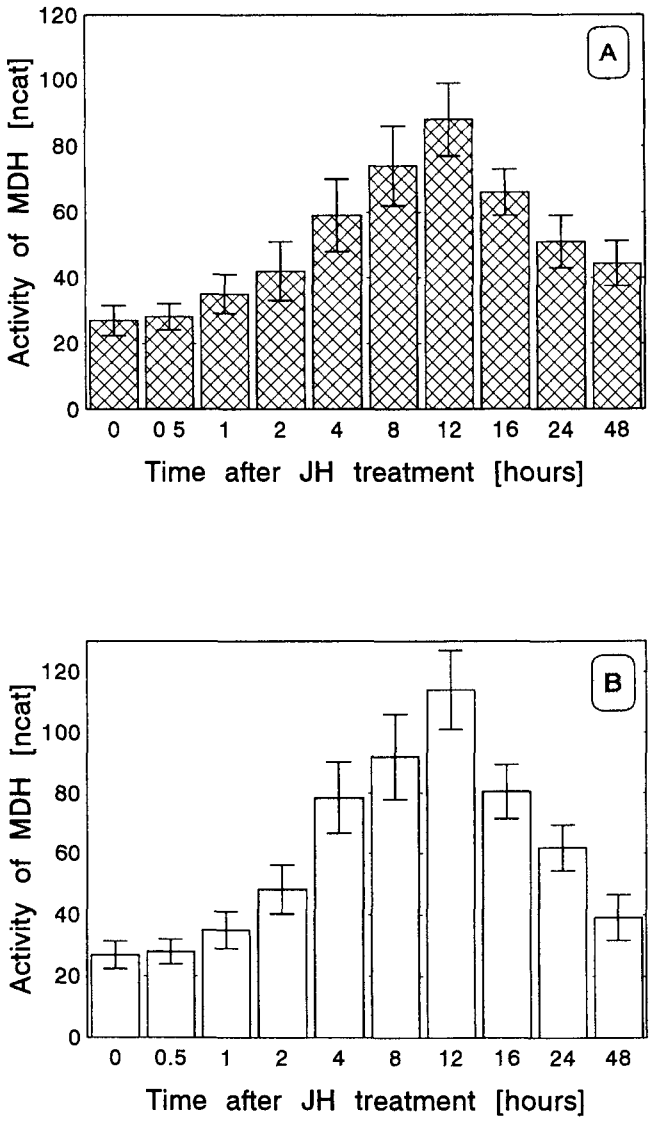

Figure 2. (*A*) JH-induced upregulation of MDH activity in feeding wild type last instar larvae JH or JHA administered 24 h prior to pupariation (*B*) The additive effect of JH on the increased MDH activity in high sucrose-fed wild type last instar larvae JH or JHA administered to 24 h prior to pupariation The elevated MDH activity starts to decline by the end of the feeding period

tivity could be detected in freshly eclosed adults (data noth shown) Nevertheless, regardless of the concentration of exogenously applied JH or JHA, and whenever applied, this hormone appears unable to rescue MDH activity in ap^4 mutant adults completely (Fig 4*A*)

Following the above findings in ap^4 adults, we also performed enzymatic assays in ap^4 larvae In ap^4 homozygous larvae of 3rd instar, which we could distinguish from heterozygotes by means of chromosomal markers for *SM*5 or *CyO* (Ashburner 1989, Lindsley and Zimm 1992), the MDH activity was found lowered by 60 to 75% in comparison to wild type controls (Fig 4*B*) In contrast to adults, this enzymatic change could be fully rescued by administration of JH or JHA (Fig 4*B*) Interestingly, also heterozygotes $ap^4/SM5$ or ap^4/CyO, displayed MDH activity lowered by 30 to 45% (Fig 4*B*) To verify that these values are not due to genetic background of balancer chromosomes, their siblings or other and viable mutations balanced over *SM*5 or *CyO* were used as controls These measurements revealed no difference from enzyme activities found in wild types (data not shown)

Discussion

Data on ecd^1 and $su(f)^{ts67g}$ mutants of *Drosophila* suggest that MDH could potentially belong to group of ecdysone-repressible enzymes (genes) as are alcohol dehydrogenase (Murtha and Cavener 1989) and urate oxidase (Wallrath et al 1990) Although this conclusion fits to our knowledge about the overall preparation of the animal for metamorphosis and seems to be valid for the regulation of larval MDH, the adult situation appears to be quite different In larvae, MDH is required to synthesize lipids as storage and energy resources to be used during the immobile pupal stage The lipogenetic process depends on the availability of carbohydrate precursors, mostly saccharides from food (Geer et al 1976, Geer and Perille 1977) which are taken in during an intense feeding period, predominantly in the first half of the last instar When the larva is ready for metamorphosis and stops feeding, lipogenesis is downregulated via repression of MDH by raising the titre of ecdysteroids Adults, especially females, as apparent from experiments with ap^4 homozygotes, require not only JH but also ecdysone for their proper reproduction This not only supports the generally accepted notion about the complex action of JH and ecdysone in insect reproduction (Mahowald and Kambysellis 1980, Koeppe et al 1985, Riddiford 1993, Bownes 1994) but may also be a plausible explanation why exogenously administered JH or JHA were unable to fully rescue adult MDH activity Even though wild type fresh adults have very low endogenous titer of ecdysone, and it raises gradually in time since it is considered as indispensable for ovarian vitellogenesis It is exemplified in ap^{ts78j}, another JH-deficient and conditional allele of *apterous* locus, in which ecdysone as well as JH titers remain low as long as restrictive conditions last (Schwartz et al 1989) Combined, these ob

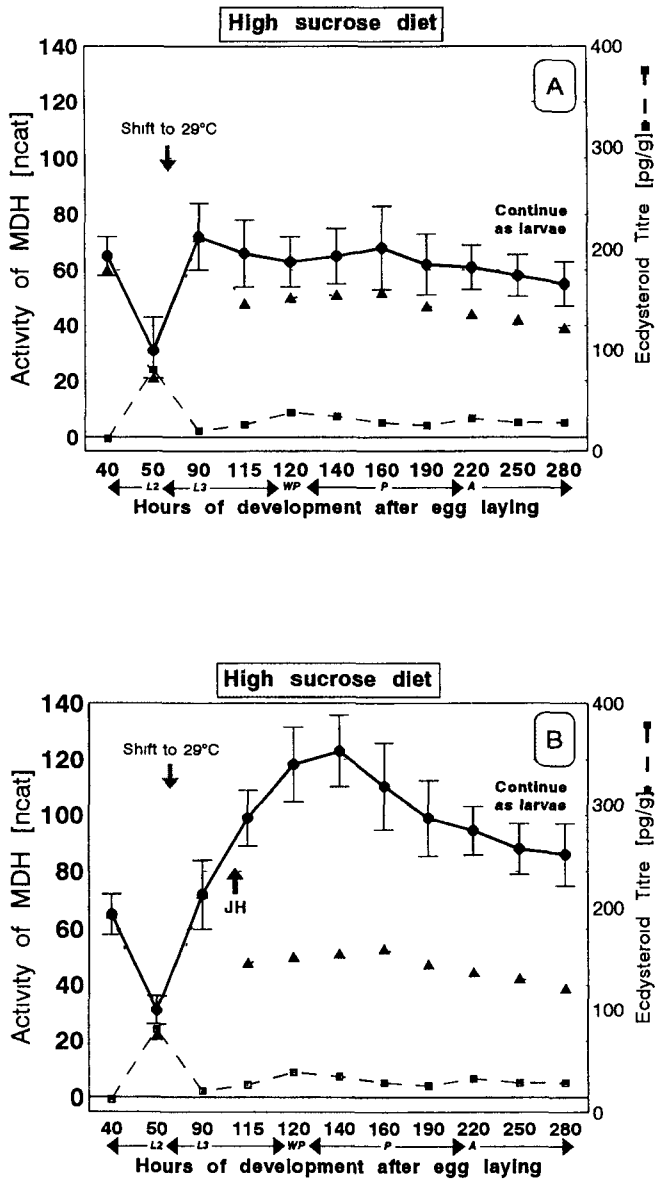

Figure 3. (*A*) Stimulation of MDH activity in larvae of ecd^1 and $su(f)^{ts67g}$ mutants by high sucrose diet Larvae maintained under restrictive conditions will not develop and continue to crowd and feed as larvae for about 10–14 days Note that the titer of the ecdysteroid hormone is very low The differences in MDH activity between the two

servations may therefore suggest that regulation of *Drosophila* MDH appears to be not only tissue- but also developmentally-specific. Similar cases have already been described and well documented for a variety of *Drosophila* enzymes including alcohol dehydrogenase (Murtha and Cavener 1989) or dopa-decarboxylase (Clark et al. 1986; Karim and Thummel 1992). Future work will have to show whether this different developmental cotrol is achieved by positive participation of ecdysone in the adult stage, but observations of increased fat body MDH in pupae of a lepidopteran insect, *Anterahea pernyi*, after ecdysone injection support this idea (Kutuzova et al. 1991).

Above we described that MDH activity of larval ap^4 homozygotes was found lowered by 60 to 75%, and this phenotype could be rescued only in part by administration of JH or JHA The substitution of JH is also capable of [1] promoting histolysis of larval fat body remnants, [2] reverting blocked synthesis and uptake of yolk proteins (vitellogenins), and subsequently [3] deblocking developmental arrest of female's vitellogenic ovaries (Postlethwait and Weiser 1973; Gavin and Williamson 1976, Tedesco et al. 1981); other defects of ap^4 phenotype, however, are not reverted by JH Therefore, what other mechanisms, besides deficiency in internal JH, might be involved in reducing MDH activity in ap^4 homozygote larvae is unclear, but we hope to address this issue in the near future by means of other *apterous* JH-deficient as well as not deficient alleles. This approach may help to exclude or at least limit additional effects of the *apterous* mutant background which is evidently pleiotropic and polyphasic (Lindsley and Zimm 1992, Shtorch et al. 1995). But at the present state of knowledge we can at least draw following conclusion. In larvae, JH does not appear to be the ultimate or key regulator of MDH activity but it seems to have additive effects besides nutritional factors that stop to act at the end of the feeding period. A higher complexity is apparent in adults where availability of nutritional factors may be dependent, in part, on their release from adult fat body after being stimulated by JH. Thus in ap^4 adults, not only JH but all the subsequent complex of reaction are missing leaving thus the adult phenotype more severe.

Results on additive increase of MDH by JH in high-sugar diet fed ecd^1 or $su(f)^{ts67g}$ mutant larvae clearly suggest that there is differential control over nu-

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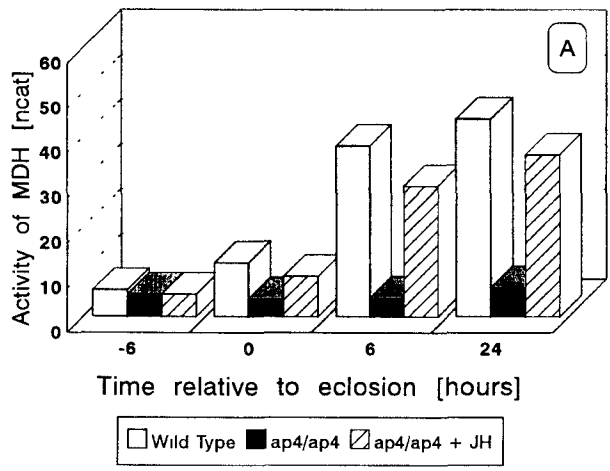
mutants were negligible and, therefore, they are plotted as a single curve in the graph The situation in normal diet-fed mutant larvae is shown as triangles (dotted line) Hours indicated under the abscissa are to indicate the corresponding developmental stages of wild types (*B*) Additional increase of MDH activity in larvae of ecd^1 and $su(f)^{ts67g}$ mutants fed high sucrose diet upon the application of JH Values around 110–130 ncat MDH are maximal we were able to elicit, this can represent maximum top values of MDH measurable in *Drosophila* larvae The MDH activity found in normally developing wild type genotype is illustrated as triangles (dotted line) Hours indicated under the abscissa are to indicate the corresponding developmental stages of wild types A=Adults, L2 and L3=2nd and 3rd larval instars, respectively, P=Pupa, WP=White Puparium



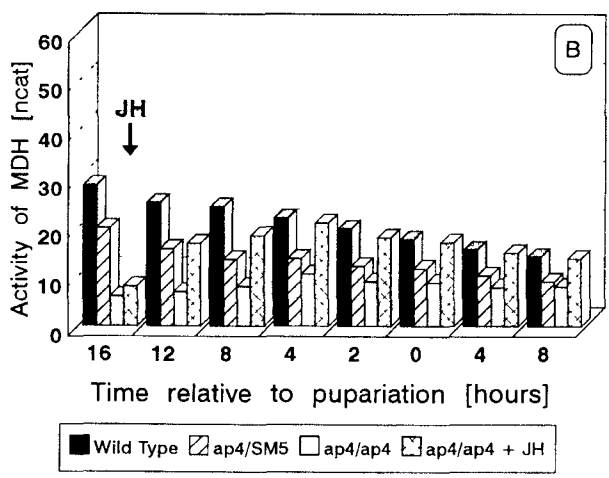


Figure 4. (*A*) Activity of MDH in adults of JH-deficient ap^4 flies. A great portion of lost MDH activity can be rescued by exogenous administration of JH or JHA on pharate adults 6 to 12 h prior to eclosion. (*B*) MDH activity in larvae of ap^4 mutation around the time of pupariation. Notice the decreased enzyme activity in $ap^4/SM5$ or ap^4/CyO heterozygotes and very low levels of MDH activity in ap^4/ap^4 homozygotes. This phenotype can be rescued by exogenous application of JH or JHA on feeding late 3rd instar larvae at least 14 h prior to pupariation as indicated by the arrow and symbol JH.

tritional and hormonal regulation of larval MDH activity in *Drosophila*, implicating a great degree of evolutionary conserved similarity between invertebrates and mammals in the regulation of lipogenetic homeostasis. Whether this regulation in *Drosophila* is also mediated via different hormonal and nutritional response elements 5′ upstream of MDH promoter, as it is, for example, in rodents (Morioka et al. 1989; Desvergne et al. 1991), remains to be elucidated. Recently, we proposed that the differential responses of *Drosophila* MDH to JH and ecdysone, especially in various developmental stages, may reflect positive regulation of different MDH isoforms or isoenzymes (Farkaš and Knopp, 1997). The concurrent and additive increase of MDH activity by JH in high sucrose-fed larvae described herein nevertheless suggest that both JH and sucrose may regulate the activity and/or the expression of the same MDH isoenzyme. Another important conclusion comes from a comparison of MDH acitivities between ecd^1 and $su(f)^{ts67g}$ which are so similar that can be considered identical. Therefore, if the genetic background has no substantial effect, then all observed changes of MDH activity in ecd^1 and $su(f)^{ts67g}$ are attributable to deficiency in the hormone ecdysone.

Regarding the response in wild type or mutant wandering larvae it should be stressed that at the time of the metamorphic onset the concentration of JH III is increased, about 1 pmol/g fresh weight (Bownes and Rembold 1987). Thus, it is not surprising that exogenous JH administration did not elicit any MDH increase in wandering stage. Inner ecdysone peak probably takes control over JH action on MDH. It needs to be stressed here that freshly eclosed normal adults have 5–7 pmol/g of JH (Bownes and Rembold 1987), a concentration 5 to 7-fold higher than the larvae. The developmental and physiological significance of this finding remains elusive. However, this might be one of several reasons why in ap^4 mutants the JH-deficient phenotype is greatly expressed in adult flies and morphogenetically not observed, or at least not apparent, in larvae. In the case of MDH, it is not a morphogenetic effect but enzyme activity and thus the effector gene can serve as an excellent challenge to explore the molecular mechanism of JH action with tools available in *Drosophila* genetics; the effort towards molecular cloning of the *Drosophila* MDH gene is currently underway in our laboratory.

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