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Phospholipid Dependence of the Reversible, Energy-Linked, Mitochondrial Transhydrogenase in *Manduca sexta*

Kurt P. Vandock · Darby J. Emerson · Kathryn E. McLendon · Alyssa A. Rassman

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Abstract Midgut mitochondria from fifth larval instar *Manduca sexta* exhibit a membrane-associated transhydrogenase that catalyzes hydride ion transfer between NADP(H) and NAD(H). The NADPH-forming transhydrogenations occur as nonenergy- and energy-linked activities. The energy-linked activities couple with electron transport-dependent utilization of NADH/succinate, or with Mg^{2+}-dependent ATPase. These energy-linked transhydrogenations have been shown to be physiologically and developmentally significant with respect to insect larval/pupal maturation. In the present study, isolated mitochondrial membranes were lyophilized and subjected to organic solvent or phospholipase treatments. Acetone extraction and addition of Phospholipase A_2 proved to be effective inhibitors of the insect transhydrogenase. Liberation of phospholipids was reflected by measured phosphorous release. Addition of phospholipids to organic solvent- and phospholipase-treated membranes was without effect. Employing a partially lipid-depleted preparation, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were reintroduced and transhydrogenase activity assessed. Of the phospholipids tested, only phosphatidylcholine significantly stimulated transhydrogenase activity. The results of this study suggest a phospholipid dependence of the *M. sexta* mitochondrial transhydrogenase.

K. P. Vandock (✉) · D. J. Emerson · K. E. McLendon · A. A. Rassman
Department of Biology, Houghton College, 307 Paine Center for Science, Houghton, NY 14744, USA
e-mail: kurt.vandock@houghton.edu

K. P. Vandock
United States Army Reserve, Army Reserve Medical Command APMC, Forest Park, GA 30297, USA

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Transhydrogenase (E.C. 1.6.1.1), isolated from wandering stage fifth larval instar *Manduca sexta* midgut tissue, is a membrane-associated enzyme that catalyzes the following reversible reaction:

$$NADPH + NAD^+ \leftrightarrow NADP^+ + NADH$$

(Vandock et al. 2008). The NADPH-forming transhydrogenation (i.e., NADH → $NADP^+$) occurs as an energy- or nonenergy-linked reaction. The energy-linked NADPH-forming reactions occur via NADH/succinate oxidation through the electron transport system or hydrolysis of ATP by a Mg^{2+}-dependent ATPase (Vandock et al. 2008). The NADH-forming activity is termed NADPH → NAD^+. This reversible transhydrogenation appears to be localized within the inner membrane of *M. sexta* mitochondria and acts as a proton translocator (Vandock et al. 2008).

Postembryonic development in *M. sexta* is dependent on the conversion of the molting hormone, ecdysone, to its physiologically active form, 20-hydroxyecdysone (20-HE) (Gilbert et al. 2002). Ecdysone 20-monooxygenase (E20-M; E.C. 1.14.99.22), a cytochrome P450-dependent steroid hydroxylase, catalyzes this crucial conversion (Smith et al. 1979; Feyereisen 2005; Lafont et al. 2005). Mitochondrial E20-M uses NADPH as the reductant, and its activity is primarily associated with tissues targeted by the ecdysone (e.g., midgut, fatbody, and Malpighian tubules) (Smith 1985; Weirich 1997; Gilbert et al. 2002).

The peak of E20-M activity in midgut mitochondria of *M. sexta* occurs on day five of the fifth larval instar (Mitchell et al. 1999). This 50-fold increase in activity corresponds to the expression of the gene *shade* that

encodes E20-M, reprogramming between the larval and pupal stages, and the beginning of the wandering stage behavior (Gilbert and Rewitz 2009). In mitochondria isolated from fatbody tissue, E20-M activity peaks on day four of the fifth larval instar (Mitchell et al. 1999). Interestingly, this increase in E20-M activity in fatbody coincides with a small peak in hemolymph ecdysteroid titer, a signal for the transition from larval tissues into pupal tissues (Lafont et al. 2005). NADPH is absolutely necessary to catalyze the mitochondrial conversion of ecdysone to 20-HE, which is key to regulating postembryonic development (Mayer et al. 1978). The *M. sexta* mitochondrial transhydrogenation serves as a source for this NADPH, as seen below (Vandock et al. 2008, 2010):

$$\mathrm{NADH + NADP^+ \xrightarrow[\text{Transhydrogenase}]{} NADPH + NAD^+}$$

$$\mathrm{NADPH + R + O_2 \xrightarrow[\text{E20-M}]{} NADP^+ + ROH + H_2O.}$$

R = ecdysone

The data that follow demonstrate that the *M. sexta* transhydrogenase and its energy-linked activities exhibit a dependence on phospholipids.

Materials and Methods

Insect Rearing and Mitochondrial Isolation

Manduca sexta were reared on an artificial diet under a nondiapausing photoperiod (L:D, 16:8) at 26°C, day, 24°C, night and ~60% relative humidity. Wandering stage animals were defined by morphological and behavioral (wandering) markers (Bell and Joachim 1976). *M. sexta* mitochondria were isolated from last instar, wandering stage (day five, gate II) larval midguts. Midguts were isolated by dissection as described by Keogh et al. (1989) and mitochondria isolated after Vandock et al. (2008). Midguts and fatbodies were homogenized in mitochondrial medium (~10.0 ml/g tissue) consisting of 250 mM sucrose, 0.15% Bovine Serum Albumin (BSA), 15 mM ethylenediaminetetracetate (EDTA) and 10 mM trishydroxymethylaminomethane-HCl (Tris–HCl, pH 7.5). Cellular debris was removed by centrifugation at 500×*g* for 10 min. Mitochondria were isolated from the resulting supernatant fraction at 10,000×*g* for 30 min and washed at 12,000×*g*. The resulting pellet was suspended in mitochondrial medium. Mitochondrial membranes were further isolated as described by Vandock et al. (2008). All steps of the mitochondrial isolations were performed at 4°C.

Assessments of Enzyme Activities

Spectrophotometric assessments of transhydrogenase were performed essentially as described by Fioravanti et al. (1992) and Vandock et al. (2008). Activities were measured by following acetylpyridine NAD(P) (AcPyAD(P)) reduction at 375 nm. For assessment of AcPyAD reduction (NADPH → NAD^+ reaction), the 1.0-ml assay system contained enzyme, and the following in µmol: Tris-HCl (pH 7.5), 100, NADPH, 0.24, AcPyAD, 0.60. Where indicated, 25 µM rotenone was present.

For assessment of AcPyADP reduction (NADH → $NADP^+$ reactions), the 1.0-ml assay system contained enzyme, 10 µg bovine serum albumin (BSA), and the following in µmol: Tris-HCl (pH 7.5), 100, NADH, 0.24, AcPyADP, 0.60. Where indicated the NADH → $NADP^+$ assays contained: 25 µM rotenone, 2.0 µM ATP, 3.0 µM $MgCl_2$, and 3.0 mM succinate. Rotenone, an inhibitor of the mitochondrial oxidase system, was dispensed in ethanolic solution, such that the ethanol content was 1.9% in the assays. Appropriate ethanol controls were performed.

NADH oxidase and succinate dehydrogenase activities were used as mitochondrial membrane markers. NADH oxidase (E.C. 1.6.3.1) was assessed by measuring NADH disappearance at 340 nm (Fioravanti 1982). The 1.0-ml assay contained enzyme, 10 µg BSA, and the following in µmol: Tris-HCl (pH 7.5), 100, NADH, 0.24. Succinate dehydrogenase (E.C. 1.3.5.1) was assessed essentially by the method of Kmetec and Bueding (1961), as described by Fioravanti (1982), by following potassium ferricyanide reduction at 410 nm. The 1.0-ml assay system contained, in addition to enzyme and 10 µg BSA, the following in µmol: Tris-HCl (pH 7.5) 100, succinate 0.24, and potassium ferricyanide 0.60.

All enzyme assessments were performed at 25°C.

Treatment of Mitochondrial Membranes

Similar to methods described by Rydström et al. (1976) membranes were lyophilized and suspended in 0.4 M Tris-HCl (pH 7.5) or treated with organic solvents essentially after the techniques of Fioravanti and Kim (1983).

Phospholipase treatments of mitochondrial membranes were performed by incubating samples with 15 units of phospholipase A_2 (bee venom, Sigma) for 20 min at 37°C.

In addition to phospholipase A_2, the 0.95-ml incubation contained 5.0 mg BSA, 100 μmol Tris-HCl (pH 7.5) and 2.5 μmol $CaCl_2$. Phospholipase incubations were terminated by addition of 0.05 ml (25 μmol) EDTA (pH 7.5). Accordingly, 25 μmol EDTA was added to control samples 5 min before incubation with phospholipase A_2.

The "30–55 Fraction" and Phospholipid Addition

To assess the effects of phospholipid addition on *M. sexta* mitochondrial transhydrogenase activity, a partially lipid-depleted preparation was used. This preparation was designated the 30–55 fraction and was attained by the methods outlined by Rydström et al. (1976), as well as Anderson and Fisher (1978). The designation 30–55 fraction was utilized because the preparation was derived from mitochondrial membranes exposed to detergent treatment and subsequent precipitation between 35–55% ammonium sulfate saturation.

Addition of phospholipids (phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine) to the 30–55 fraction was performed after the protocol established by Fioravanti and Kim (1983). Dispersed by sonication in the presence of nitrogen, aliquots of the 30–55 fraction were added to the sonicated lipid. The methods used for *Hymenolepis diminuta* (Fioravanti and Kim 1983) were modified slightly to include two 1-min sonication treatments with 4-min intervals between each sonication. All steps of lipid addition were performed at 4°C.

Protein and Inorganic Phosphorous Assessment

Protein content of mitochondrial preparations was determined by the method of Bradford (1976) using BSA as the standard. Inorganic phosphorous content was determined by the method established by Fiske and Subbarow (1925).

Materials

BSA, disodium succinate, and Tris-HCl were purchased from Fisher Scientific Co. (Waltham, MA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). *M. sexta* eggs were purchased from Carolina Biological, Burlington, NC, and specimens were obtained using the methods described by Smith et al. (1979) for the growth and staging of tobacco hornworms.

Results

Reversible Transhydrogenations Catalyzed by *Manduca sexta* Mitochondria

Table 1 presents the reversible transhydrogenase activities catalyzed by mitochondrial membranes isolated from midgut tissue of wandering stage, fifth larval instar *M. sexta.* Wandering stage larva from the fifth larval instar were utilized for all assessments because peak transhydrogenase and E20-M activity is noted during this crucial developmental period (Vandock et al. 2010). As presented in Table 1, the NADPH → NAD^+ reaction displayed the highest activity. For the NADH → $NADP^+$ transhydrogenations, activity measured in the presence of rotenone was deemed nonenergy linked, consistent with rotenone inhibition of the mitochondrial oxidase system. Conversely, in the absence of rotenone, energy-linked NADH → $NADP^+$ transhydrogenation resulting from electron transport–dependent NADH oxidation was apparent and is termed the ETD reaction (Table 1). Furthermore, succinate-dependent activity (the SD reaction) in the presence of rotenone was apparent with a fivefold stimulation over that noted for the nonenergy-linked activity. The greatest stimulation of the NADH → $NADP^+$ transhydrogenation was noted in the presence of rotenone, ATP and Mg^{2+}. This activity is driven by ATP hydrolysis via Mg^{2+}-dependent ATPase and is termed the ATPD reaction. All assessments of energy-linked NADH → $NADP^+$ activity were corrected for the nonenergy-linked reaction.

Table 1 Transhydrogenations catalyzed by *Manduca sexta* midgut mitochondrial membranes

Reaction	Addition	Activity (μmol min^{-1} [mg protein]$^{-1}$)
NADPH → NAD^+ NADH → $NADP^+$	Rotenone (25 μM)	0.71 ± 0.046 (22)
Nonenergy linked	Rotenone	0.03 ± 0.008 (18)
ETD		0.12 ± 0.013 (18)
SD	Rotenone, succinate (3.0 mM)	0.17 ± 0.020 (18)
ATPD	Rotenone, $MgCl_2$ (3.0 mM), ATP (2.0 mM)	0.36 ± 0.051 (18)

Values are observation means ± SE; number of observations is in parentheses; observations were made from three separate mitochondrial extractions. A total of 0.24 mg protein was used for assays. All assessments of energy-linked NADH → $NADP^+$ activity were corrected for the nonenergy-linked reaction

Lyophilization and Treatment of Mitochondrial Membranes with Organic Solvents

After lyophilization, all transhydrogenations catalyzed by the treated *M. sexta* mitochondrial membranes were subjected to organic solvent treatment and the activities were assessed. Every transhydrogenation responded similarly to treatments; therefore, the reactions with the highest activities (i.e., the NADPH → NAD^+ and ATPD NADH → $NADP^+$ reactions) were chosen as representative samples

Table 2 Effects of organic solvent and phospholipase treatments on transhydrogenase activities in *Manduca sexta* mitochondrial membranes

Treatment	Activity (μmol min^{-1} [mg protein]$^{-1}$)	
	NADPH → NAD^+	ATPD: NADH → $NADP^+$
None	0.71 ± 0.046 (22)	0.36 ± 0.051 (18)
Lyophilization (LY)	0.66 ± 0.069 (10)	0.34 ± 0.068 (18)
LY, hexane (HA)	0.72 ± 0.057 (12)	0.37 ± 0.062 (12)
LY, HA, 99% acetone (AC)	0.13 ± 0.024 (8)	0.06 ± 0.019 (8)
LY, HA, 95% AC	0.09 ± 0.023 (8)	0.04 ± 0.014 (8)
LY, HA, 90% AC	0.10 ± 0.037 (8)	0.05 ± 0.011 (8)
EDTA plus phospholipase A_2[a]	0.65 ± 0.039 (12)	0.31 ± 0.018 (12)
Phospholipase A_2	0.08 ± 0.013 (8)	0.05 ± 0.034 (8)

Values are observation means ± SE; number of observations is in parentheses. A total of 0.20 mg protein was used for assays. When present, rotenone, $MgCl_2$, and ATP concentrations were 25 μM, 3.0, and 2.0 mM, respectively

[a] EDTA was included in a 5-min preincubation of the assay. After this incubation, phospholipase A_2 (15 units) was added and assessment completed

for data presentation. As detailed in Table 2, lyophilization of mitochondrial membranes resulted in a slight decrease in insect transhydrogenase activity.

Utilizing lyophilized membranes, hexane extraction of both the NADPH → NAD^+ and ATPD transhydrogenations resulted in a restoration of activity similar to that noted with no treatment (Table 2). Subsequent extraction with 99% acetone significantly diminished both transhydrogenase activities. For both the NADPH → NAD^+ and the ATPD transhydrogenations, the greatest reduction in transhydrogenase activity was noted with 95% acetone (88 and 89% inhibition, respectively). Treatment of hexane-extracted membranes with 90% acetone did not significantly alter the degree of inhibition noted for the 95% acetone extraction. These data suggest that the demonstrated aqueous acetone-dependent extraction of phospholipids directly contributes to significantly reduced transhydrogenase activity. Furthermore, when the organic solvent–treated lyophilized membranes were assessed for phosphorous content, only the acetone-treated samples displayed significant amounts of released phosphorous.

Treatment of Mitochondrial Membranes with Phospholipase A_2

Isolated mitochondrial membranes were also subjected to treatment with phospholipase A_2 (bee venom). In keeping with an apparent phospholipid dependence, the NADPH → NAD^+ and ATPD transhydrogenase activities were inhibited significantly by phospholipase A_2, (89 and 86%, respectively; Table 2). Controls were performed with a preincubation that included sufficient EDTA to inactivate the phospholipase. These controls established that incubation with phospholipase was necessary for the reported reduction in activities.

Phospholipid Addition to a Partially Lipid-Depleted Preparation

Phospholipids (viz., phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine) were added to organic solvent or phospholipase treated membranes, and no significant stimulation or restoration of transhydrogenase activity was demonstrable. Accordingly, a partially lipid-depleted preparation was used. This preparation designated the 30–55 fraction, was derived from mitochondrial membranes as detailed in Materials and Methods 2.4. The phospholipid contents of mitochondrial membranes and the 30–55 fraction were determined by measurement of phosphorous/mg protein (Table 3). Indeed, a 65% decrease in phosphorous content/mg protein was noted after purification of the 30–55 fraction, indicative of a partially lipid-depleted preparation.

With respect to phospholipid addition to the 30–55 fraction, each transhydrogenation responded similarly to treatments. As a result, the NADPH → NAD^+ was used as a representative sample for data presentation in Table 4. While preparations of the 30–55 fraction contained less protein (50%) than mitochondrial membrane extracts, the activities of all transhydrogenations were not significantly altered by the methods used to reintroduce phospholipids to this fraction. Interestingly, addition of phosphatidylcholine to the 30–55 fraction significantly stimulated *M. sexta* mitochondrial transhydrogenations. The greatest stimulation noted was with supplementation of 0.50 μmol phosphatidylcholine. Further accumulation of phosphatidylcholine in excess of 0.50 μmol did not significantly affect transhydrogenase activities. Both phosphatidylethanolamine and phosphatidylserine did not significantly stimulate transhydrogenase activity at any concentration. To ensure that stimulation of the transhydrogenase in the 30–55 fraction was a result of phospholipid introduction and not an artifact of detergent treatment, cholate and deoxycholate were assessed at the stimulatory levels noted for phosphatidylcholine, with no significant effect. Finally, untreated

Table 3 Phosphorus content of *Manduca sexta* mitochondrial membranes and the partially lipid-depleted 30–55 fraction

Preparation	μg phosphorus/mg protein
Mitochondrial membranes	38.3
30–55 fraction[a]	12.1

[a] Preparation of the 30–55 fraction is described in text

Table 4 Effects of phospholipid addition on the NADPH → NAD^+ transhydrogenase activity from the 30–55 fraction of *Manduca sexta*

Phospholipid	Amount (μmol)	Activity (μmol min^{-1} [mg protein]$^{-1}$)
Phosphatidylcholine	None[a]	0.56 ± 0.032 (10)
	0.25	0.71 ± 0.074 (8)
	0.50	0.81 ± 0.086 (8)
	1.0	0.79 ± 0.101 (8)
Phosphatidylethanolamine	None	0.55 ± 0.036 (10)
	0.25	0.58 ± 0.073 (8)
	0.50	0.61 ± 0.084 (8)
	1.0	0.61 ± 0.091 (8)
Phosphatidylserine	None	0.53 ± 0.039 (10)
	0.25	0.56 ± 0.066 (8)
	0.50	0.62 ± 0.088 (8)
	1.0	0.65 ± 0.098 (8)

Activities are observation means ± SE; number of observations is in parentheses. A total of 0.14 mg protein was used for assays

[a] Material was subjected to similar treatment noted for samples with phospholipid addition

mitochondrial membranes were subjected to phosphatidylcholine addition and were unable to produce the activation distinguished by the 30–55 fraction.

Discussion

Mitochondrial transhydrogenase plays a key role in larval-pupal development of *M. sexta* (Vandock et al. 2010). This enzyme is a reversible, membrane-associated, mitochondrial component, and acts as a transmembrane proton translocator (Vandock et al. 2008). During the ten day developmental period preceding the larval-pupal molt (fifth larval instar), mitochondrial transhydrogenase activities of midgut and fatbody tissues are greatest during the onset of wandering behavior. This peak of activity coincides with a 50-fold increase in NADPH-requiring E20-M (Smith et al. 1979; Mitchell et al. 1999; Vandock et al. 2010). The energy-linked reactions, representing a reversal of the NADPH → NAD^+ transhydrogenation, are driven by electron transport-dependent NADH or succinate oxidation (ETD or SD) or by Mg^{2+}-dependent ATP hydrolysis (ATPD). These transhydrogenations result in the accumulation of NADPH necessary to convert ecdysone to 20-HE for insect maturation.

In the present study, phospholipid dependence of the *M. sexta* mitochondrial transhydrogenase was suggested by organic solvent or phospholipase treatments. Although hexane extraction had no effect on the lyophilized mitochondrial membranes, consecutive aqueous evulsion with acetone effectively diminished transhydrogenase activities, with maximal inhibition noted at 95% acetone. This decrease in transhydrogenase is consistent with liberation of phospholipids as established by measurement of phosphorous content from material extracted with acetone. Further evidence to support the necessity of phospholipids for optimal transhydrogenase function was demonstrated by incubation of mitochondrial membranes with phospholipase A_2. When incubated with phospholipase A_2, mitochondrial membranes displayed decreased transhydrogenase activity. Interestingly, phospholipid addition to either organic solvent or phospholipase treatments did not result in enhanced transhydrogenase activity. This may be the result of direct denaturation of the transhydrogenase from organic solvent treatment or inactivation of the transhydrogenase by phospholipase A_2 resulting from conversion of membrane lipids to arachidonic acid and lysophospholipids.

When the partially lipid-depleted 30–55 fraction was used, phospholipid addition enhanced transhydrogenase activity. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were all reintroduced to the 30–55 fraction; however, only phosphatidylcholine was effective at stimulating transhydrogenase activity. Notably, with the 30–55 fraction, there was a 20% enhancement of transhydrogenase activity with phosphatidylcholine as compared to mitochondrial membranes.

The phospholipid dependence of mitochondrial transhydrogenase has been noted in other systems. *Escherichia coli*, *Hymenolepis diminuta*, and bovine heart transhydrogenase have all demonstrated phospholipid dependence (Rydström et al. 1976; Houghton et al. 1976; Anderson and Fisher 1978; Fioravanti and Kim 1983; Eytan et al. 1987). In these systems, it was observed that lipid-depleted preparations were significantly stimulated by phosphatidylcholine.

In *M. sexta*, the only phospholipid that effectively stimulated the transhydrogenase activity of the 30–55 fraction was phosphatidylcholine. In contrast to reports regarding mammalian and bacterial transhydrogenases, the insect enzyme was unaffected by phosphatidylethanolamine (Rydström et al. 1976; Houghton et al. 1976). The results from the present study are similar to those noted for the invertebrate, *H. diminuta*, that displayed specific dependence for phosphatidylcholine (Fioravanti and Vandock 2010). The effects of other phospholipids or combinations of lipids on the *M. sexta* transhydrogenase requires further investigation. Nevertheless, the results we detail here clearly demonstrate a phospholipid dependency of the *M. sexta* transhydrogenase and set the framework for future studies aimed at further characterization of the energy-linkages and membrane dynamics of this important mitochondrial system.

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