Control of oxidative phosphorylation during insect metamorphosis

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Chamberlin, M. E. Control of oxidative phosphorylation during insect metamorphosis. Am J Physiol Regul Integ Comp Physiol 287: R314–R321, 2004. First published April 8, 2004; 10.1152/ajpregu.00144.2004.—The midgut of the tobacco hornworm (Manduca sexta) is a highly aerobic tissue that is destroyed and replaced by a pupal epithelium at metamorphosis. To determine how oxidative phosphorylation is altered during the programmed death of the larval cells, top-down control analysis was performed on mitochondria isolated from the midguts of larvae before and after the commitment to pupation. Oxygen consumption and protonmotive force (measured as membrane potential in the presence of nigericin) were monitored to determine the kinetic responses of the substrate oxidation system, proton leak, and phosphorylation system to changes in the membrane potential. Mitochondria from precommitment larvae have higher respiration rates than those from postcommitment larvae. State 4 respiration is controlled by the proton leak and the substrate oxidation system. In state 3, the substrate oxidation system exerted 90% of the control over respiration, and this high level of control did not change with development. Elasticity analysis, however, revealed that, after commitment, the activity of the substrate oxidation system falls. This decline may be due, in part, to a loss of cytochrome c from the mitochondria. There are no differences in the kinetics of the phosphorylation system, indicating that neither the F₁F₀ ATP synthase nor the adenine nucleotide translocase is affected in the early stages of metabolism. An increase in proton conductance was observed in mitochondria isolated from postcommitment larvae, indicating that membrane area, lipid composition, or proton-conducting proteins may be altered during the early stages of the programmed cell death of the larval epithelium.

Manduca sexta; elasticity analysis; midgut; programmed cell death; cytochrome c

Larval Lepidoptera (caterpillars) are essentially “eating machines” and grow faster than young mammals and birds (44). One species, the tobacco hornworm (Manduca sexta), increases its mass 10,000-fold in ~16 days, and >80% of this growth occurs in the final larval instar (26). This phenomenal growth rate is due, in part, to the large midgut epithelium, which digests and absorbs nutrients. In addition, this epithelium transports ions at high rates (12, 17, 52) and elaborates a very alkaline secretion (13, 19, 38). The midgut epithelium has little capacity for anaerobic metabolism (11, 14, 24); therefore, when it is made anoxic (21) or exposed to cyanide (36), active transport drops to nil. Because of its reliance on aerobic metabolism, any changes in mitochondrial function might be expected to affect epithelial ion transport. Such changes are observed during the early stages of larval-pupal metamorphosis, when epithelial ion transport and mitochondrial respiration are depressed (14, 16).

Although it is clear that active ion transport is dependent on aerobic metabolism, it does not appear that aerobic metabolism is affected by the rate of active ion transport. Inhibition of active ion transport in the tobacco hornworm midgut produces no change in tissue respiration (36). This is in contrast to the observation in most cells that inhibition of active ion transport results in a drop in oxygen consumption as mitochondria decrease their rate of ATP production in the face of a decreased ATP turnover (18). This apparent lack of respiratory control of mitochondria in the midgut tissue is not due to the absence of ion-motive ATPases or the presence of uncoupled mitochondria. Mitgut active ion transport is thought to be energized by a V-type ATPase (51), and Mandel et al. (37) demonstrated that ATP appears to be the source of energy for active ion transport in the midgut. In addition, respiration of isolated midgut mitochondria is stimulated by ADP (11), indicating that the mitochondrial respiration is coupled to ATP synthesis. These studies, however, do not reveal the extent to which midgut mitochondrial respiration is controlled by the phosphorylation status. Given that control of oxidative phosphorylation is quite complex and distributed (40, 41), it is possible that other factors play a more important role in controlling the respiration of midgut mitochondria.

The present study employs top-down control analysis to describe the control of oxidative phosphorylation of tobacco hornworm midgut mitochondria. This method has been previously used to study isolated mammalian (29, 46) and plant (33) mitochondria as well as mitochondria within isolated mammalian (31) and molluscan (3) cells. This approach involves conceptually dividing oxidative phosphorylation into three blocks of reactions (subsystems) that are linked by a single intermediate, the protonmotive force (Δp). One subsystem, the “substrate oxidation system,” encompasses all the processes (metabolite transport, citric acid cycle, and electron transport chain) that produce Δp. The other two subsystems, “proton leak” (includes all cation cycles) and “phosphorylation system” (includes inorganic phosphate transport, adenine nucleotide translocase, and F₁F₀ ATP synthase), dissipate Δp. Because the blocks share a common intermediate, perturbation of one block will affect Δp and thus alter flux through the other block(s). The kinetic response (oxygen consumption) of each block to changes in Δp is determined, and the elasticities of each subsystem can then be used to calculate flux control coefficients (29).

The data obtained to perform top-down control analysis can also be used for elasticity analysis, which allows identification of the subsystem(s) that is(are) modulated by an external effector, such as toxins, hormones, temperature, or physiological insults (reviewed in Ref. 6). This approach, however, has

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not been used to analyze how oxidative phosphorylation is altered during animal development. The present study employs elasticity analysis to identify the components of oxidative phosphorylation that are responsible for the previously observed (14, 16) depression of midgut mitochondrial metabolism during larval-pupal metamorphosis in the tobacco hornworm. The larval midgut is destined to be destroyed at metamorphosis (47), and the results of the present study point to a substantial decrease in the activity of the substrate oxidation system in mitochondria isolated from midguts that are in the early stages of death. A feature common to many cells undergoing programmed death is the release of cytochrome c from the outer surface of the inner mitochondrial membrane (28), where it normally shuttles electrons between complex III and complex IV. The present study measures the mitochondrial levels of cytochrome c as well as cytochrome aa3 (cytochrome c oxidase) to determine whether changes in these components of the substrate oxidation system are associated with the metamorphic decline in the activity of this subsystem of oxidative phosphorylation.

MATERIALS AND METHODS

Insects. Approximately 4 days after the molt to the fifth and final instar, there is a small rise in hemolymph ecdysteroids (53), known as the “commitment peak.” This pulse of ecdysteroids does not induce ecdisis but does initiate the process of metamorphosis (reviewed in Ref. 42). The present study uses precommitment, as well as postcommitment, larvae to study midgut mitochondrial metabolism during the early stages of metamorphosis. M. sexta larvae were raised from eggs obtained from a colony at Ohio University. Larvae were fed an artificial diet (no. 9783, BioServ, Frenchtown, NJ) and maintained at 25°C on a 16:8-h light-dark cycle. Larvae were staged by the method described by Chamberlin et al. (15). Day 2 larvae were ~60 h beyond the molt to the fifth instar, and those weighing 3–6.5 g were used in the present study. Day 4 larvae, which were ~108 h beyond the molt to the fifth instar, were used if they weighed ≥8 g but displayed no visible effects of the commitment peak (e.g., cessation of feeding, an empty gut, a blue dorsal vessel, and increased locomotory behavior). It was assumed, however, that these day 4 larvae had experienced the commitment peak of ecdysteroids, because titers begin to rise ~84 h after the molt to the fifth instar (53). In contrast to day 4 larvae, day 5 (wandering stage) overtly manifested the effects of the commitment peak listed above.

Isolation of mitochondria. Midguts were dissected from 6–18 larvae, and the mitochondria were isolated as previously described (24), except the entire midgut, rather than just the posterior section, was used. Mitochondria from the different midgut sections have similar substrate preferences and substrate oxidation rates (11). The final mitochondrial pellet was suspended in isolation medium (24), and the protein content was measured (4) and adjusted to 10 mg/ml.

Top-down analysis. Top-down analysis involves monitoring the kinetic response (measured as oxygen consumption) of the proton leak, the substrate oxidation system, and the phosphorylation system to their common intermediate, Δp. In the present study, Δp was measured in the presence of nigericin, so that Δp is expressed entirely as membrane potential. Preliminary studies showed that the dose of nigericin used in this study was sufficient to fully hyperpolarize the mitochondrial membrane potential, yet not inhibit mitochondrial respiration. Because redox slip (electron flow without proton pumping) has been shown to be negligible in animal (7) mitochondria, it was assumed that the rate of oxygen consumption is directly proportional to the rate at which protons are pumped by the electron transport chain. Even if redox slip takes place, it will not affect the conclusions based on the top-down analysis (8).

Fig. 1. Representative recordings of oxygen uptake and triphenylmethylphosphonium (TPMP+) concentration during titration of the subsystems of oxidative phosphorylation in midgut mitochondria isolated from day 2 larvae. A: addition of malonate (top arrows; final concentrations 0.7, 1.4, 2.1, 3.5, 4.9, 6.3, 7.6, and 9.0 mM) to state 4 mitochondria to state 4 mitochondria to determine kinetics of proton leak. B: addition of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (top arrows; final concentrations 0.3, 1.0, 1.7, 2.4 mM) to state 4 mitochondria to determine kinetics of substrate oxidation system. C: Loss of mitochondrial TPMP+ when anoxia was reached. C: addition of malonate (top arrows; final concentrations 0.2, 0.3, 1.0, 1.4, 1.7, 2.4, and 3.8 mM) to state 3 mitochondria to determine kinetics of phosphorylation system.
Measurement of mitochondrial oxygen consumption and membrane potential. A miniature Clarke-type oxygen electrode (Instech Laboratories, Plymouth Meeting, PA) and a methyltriphénylphosphonium (TPMP\(^{+}\)) electrode were inserted into a temperature-controlled (25°C) respiration chamber to measure oxygen consumption and membrane potential simultaneously. The oxygen electrode was calibrated by first equilibrating water in the respiration chamber with room air and subsequently removing the dissolved oxygen with a few crystals of sodium dithionite. The signal from the oxygen electrode was amplified with an Instech model 203 amplifier. Atmospheric pressure was recorded during calibration, and the oxygen concentration of water equilibrated to 25°C was calculated using Dalton’s law of partial pressures, Henry’s law, and the oxygen solubility coefficient reported by Cameron (10).

A TPMP\(^{+}\) electrode was constructed by draping a TPMP\(^{+}\)-sensitive membrane (5) over a hollow Plexiglas electrode body, securing the membrane with an O-ring, and filling the electrode with electrode solution (in mM: 120 KCl, 1 EGTA, 10 HEPES, and 10 TPMP\(^{+}\), pH 7.2). A platinum wire was inserted into the back of the electrode, and the signal was amplified by a preamplifier (model 302, Sable Systems). A calomel electrode, which was connected to the solution in the respiration chamber via an agar bridge (4% agar and 3 M KCl) served as the reference electrode.

Reaction medium (120 mM KCl, 50 mM sucrose, 10 mM HEPES, 10 mM KH\(_2\)PO\(_4\), 1 mM MgCl\(_2\), 1 mM EGTA, 10 mM glucose, 10 mM sodium succinate, 123 nM nigericin, 20 \(\mu\)M rotenone, and 1% essentially fatty acid-free bovine serum albumin, pH 7.2) was added to the chamber, and five aliquots of TPMP\(^{+}\) were added to calibrate the TPMP\(^{+}\) electrode. The mitochondrial suspension was added to the chamber to achieve 10.25-fold dilution (final mitochondrial concentration 0.975 mg protein/ml). The total TPMP\(^{+}\) concentration was 24.7 \(\mu\)M, and preliminary experiments showed that this concentration of TPMP\(^{+}\) did not inhibit or uncouple the mitochondria (data not shown). The chamber was sealed, and the oxygen consumption and extramitochondrial TPMP\(^{+}\) concentration were recorded using a computer-based data acquisition system (Datacan V, Sable Systems). The extramitochondrial TPMP\(^{+}\) concentration detected by the electrode reflects the total concentration of TPMP\(^{+}\) minus any TPMP\(^{+}\) that had entered the matrix and/or was bound by the mitochondria. With the use of a method similar to that of Lotscher et al. (35), the amount of TPMP\(^{+}\) bound by the mitochondria was determined by subtraction of the extramitochondrial TPMP\(^{+}\) concentration in the presence of anoxic mitochondria from the total TPMP\(^{+}\) concentration. The amount of TPMP\(^{+}\) bound did not differ among mitochondrial preparations made from midguts of larvae at different stages of development. The matrix TPMP\(^{+}\) concentration was determined by dividing the amount of TPMP\(^{+}\) that entered the mitochondria by the matrix volume (see below). The extra- and intramitochondrial TPMP\(^{+}\) concentrations were used to calculate the membrane potential with the Nernst equation.

Measurement of mitochondrial volumes. Mitochondria were incubated in reaction medium plus 0.1 mM mannitol, [\(^{14}\)C]mannitol, and \(^{3}\)H\(_2\)O for 2 min at 25°C. The suspension was then centrifuged at 12,000 g for 2 min, and the supernatant was discarded. The \(^{14}\)C and \(^{3}\)H activities in the pellet were measured, and the extramitochondrial ([\(^{14}\)C]mannitol) space was subtracted from the total mitochondrial (\(^{3}\)H\(_2\)O) space to yield the matrix volume. The matrix volume did not differ among the mitochondria isolated from larvae at different stages of development. In addition, there was no difference in the matrix volume of state 4 and state 3 mitochondria. Therefore, the same value, 1.03 \(\mu\)l/mg protein, was used in all calculations.

Kinetic responses of the subsystems. The kinetic responses of all systems were determined in the presence of 0.5 mM ADP. The kinetics of the proton leak were determined in the presence of 2.8 \(\mu\)g/ml oligomycin to inhibit ATP production. Small doses (0.7–9.0 mM) of malonate were sequentially added, resulting in the inhibition of oxygen consumption and the elevation of the extramitochondrial TPMP\(^{+}\) concentration as the mitochondria depolarized (Fig. 1A). The kinetics of the substrate oxidation system were determined by sequential additions of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (0.3–2.4 \(\mu\)M) in the presence of oligomycin. These maneuvers resulted in a stimulation of oxygen consumption as the mitochondria depolarized (Fig. 1B). Titration of the phosphorylation system was performed in the presence of hexokinase (10 U/ml) to maintain a constant state 3 rate. Malonate (0.2–3.8 mM) was added sequentially,

![Fig. 2](image-url)
resulting in a depression of oxygen consumption and depolarization of the mitochondria (Fig. 1C). The oxygen consumption due to the proton leak was subtracted from the state 3 rate at any given membrane potential to report only the kinetic response of the phosphorylation system.

**Cytochrome concentrations.** Midgut tissue was dissected from the larvae, weighed, and gently homogenized in isolation medium. Some of the homogenate was reserved, and one part 4% Triton X-100 was added to three parts of the homogenate. Mitochondria were isolated from the remaining homogenate and diluted 25% with 4% Triton X-100. The samples sat on ice for 10 min and then were centrifuged

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**Fig. 3.** Kinetics of subsystems of oxidative phosphorylation in mitochondria isolated from larvae at different stages of development. A: proton leak. B: substrate oxidation system. C: phosphorylation system. Values are means ± SE for 6 mitochondrial preparations; error bars smaller than symbol are not shown.

**Fig. 4.** Activities of oxidative phosphorylation subsystems at 160 mV. A: proton leak. B: substrate oxidation system. C: phosphorylation system. Values were calculated from regression equations generated for each mitochondrial preparation and then averaged.
at 1,000 g for 2 min to pellet any insoluble material. The cytochrome hemes were detected by monitoring the reduced (dithionite present) minus oxidized (ferricyanide present) difference spectra. Extinction coefficients for the wavelength pairs 550–535 nm (cytochrome c) and 605–630 nm (cytochrome a/a3), reported by Schneider et al. (48), were used to calculate cytochrome concentrations. Cytochrome aa3 was calculated by dividing the cytochrome a/a3 value by 2.

Calculations and statistics. The data from the kinetic analyses were fitted by third-order polynomial regressions using Excel. The regression equations were used to calculate oxygen consumption at different membrane potentials. In addition, elasticities were calculated from the first derivative, and flux control coefficients were calculated from the elasticities as described by Brand et al. (8) and Hafner et al. (29). Differences between oxygen consumption, membrane potential, or cytochrome content measured in preparations isolated from larvae at different stages of development were analyzed using a one-way ANOVA followed by a Tukey-Kramer multiple comparisons test. In all instances, \( P < 0.05 \) was considered to represent a significant difference.

RESULTS

Characteristics of the mitochondrial preparations. Mitochondria isolated from day 2 larvae have a state 4 rate that is 1.6- and 1.7-fold greater than the respective rates in mitochondria isolated from day 4 and day 5 larvae, respectively (Fig. 2A). The state 3 rate of day 2 mitochondria is 2 and 2.4 times

### Table 1. Mitochondrial proton flux and conductance at 160 mV

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Proton Flux, nmol H⁺·min⁻¹·mg protein⁻¹</th>
<th>Proton Conductance, nmol H⁺·min⁻¹·mg protein⁻¹·mV⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>50.1</td>
<td>0.31</td>
</tr>
<tr>
<td>Day 4</td>
<td>36.5</td>
<td>0.23</td>
</tr>
<tr>
<td>Day 5</td>
<td>41.6</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Proton flux was calculated assuming H⁺-to-O₂ ratio of 12. Values were calculated from regression equations for each preparation and then averaged.

![Fig. 5. Control of mitochondrial respiration exerted by the 3 subsystems of oxidative phosphorylation. A: state 4 respiration. B: state 3 respiration. Flux control coefficients were determined for each mitochondrial preparation and then averaged.](http://ajpregu.physiology.org/)
greater than the rates of day 4 and day 5 mitochondria, respectively (Fig. 2B). In addition, the membrane potential of day 2 mitochondria is slightly, but significantly, higher than that of day 5 mitochondria in state 3 (Fig. 2D) and higher than that of day 4 and day 5 mitochondria in state 4 (Fig. 2C).

Kinetic responses of subsystems. Figure 3 shows the kinetic responses of the three subsystems to the membrane potential. In addition, Fig. 4 compares the flux through these subsystems at 160 mV, a membrane potential within the range of measurement of the kinetic responses of all three subsystems in mitochondria isolated from larvae at all three stages of development. Mitochondria isolated from larvae at different stages of development displayed a nonlinear relation between the proton leak rate and membrane potential (Fig. 3A). This result indicates that proton conductance increases with membrane potential and is maximal in state 4. Proton conductance at the state 4 membrane potential is highest in mitochondria isolated from larvae at all three stages of development (Fig. 3B). No significant differences in proton conductance between day 2 and day 4 mitochondria (0.81 and 0.57 nmol H⁺·min⁻¹·mg protein⁻¹·mV⁻¹, respectively) were calculated at the highest membrane potential achieved by day 5 mitochondria (average 193 mV; Fig. 2C), the day 5 mitochondria have a higher proton conductance (1.33 ± 0.03 nmol H⁺·min⁻¹·mg protein⁻¹·mV⁻¹).

The maximal rate of uncoupled respiration was higher in day 2 than in day 4 and day 5 mitochondria: 101.8 ± 4.5 vs. 54.8 ± 1.9 and 47.7 ± 3.2 nmol O₂·min⁻¹·mg protein⁻¹, respectively (Fig. 3B). The data in Figs. 3B and 4B clearly indicate that, at any membrane potential, the flux through the substrate oxidation system is higher in mitochondria from day 2 than from day 4 and day 5 larvae.

Although the mitochondria from day 2 larvae had a higher rate of state 3 respiration than those from day 4 and day 5 larvae (Fig. 2B), overall, the kinetics of the phosphorylation system are similar in mitochondria isolated from larvae at all stages of development. In addition, when the kinetic response of the phosphorylation system is compared at 160 mV, there is no difference among the mitochondrial preparations (Figs. 3C and 4C).

Flux control coefficients. Figures 5 and 6 show the flux control coefficients in mitochondria isolated from day 2, day 4, and day 5 larvae. In state 4, the control over respiration is shared between the proton leak and substrate oxidation system (Fig. 5A), and there appears to be a trend for the substrate oxidation system to confer more control over the system in day 4 and day 5 larvae. In state 3, the substrate oxidation system confers most of the control over oxygen consumption in all stages of development (Fig. 5B). The substrate oxidation system also confers most of the control over the flux through the phosphorylation system (Fig. 6A). The proton leak confers most of the control over flux through the proton leak system (Fig. 6B). The negative control over the proton leak conferred by the phosphorylation system reflects the fact that increased flux through phosphorylation would decrease flux through the proton leak pathway (23).

Cytochrome content. Figure 7 shows a typical difference spectra for isolated mitochondria and tissue homogenates. Cytochrome contents were higher in the mitochondrial preparations than in the homogenates, reflecting the enrichment by the mitochondrial isolation procedure. Cytochrome c content was significantly higher in mitochondria isolated from day 2 than from day 4 and day 5 larvae (Table 2). On the other hand, cytochrome aa₃ content was the same in mitochondria from day 2 and day 4 larvae. On the basis of the cytochrome a + a₃ content, it is possible to estimate the mitochondrial protein content of the midgut tissue. Mitochondrial protein content was significantly lower in midguts from day 5 than from day 4 or day 2 larvae (32.2 ± 9.8 vs. 52.1 ± 10.0 and 70.0 ± 8.5 mg protein/g wet wt, respectively, n = 5), indicating that mitochondria begin to disappear from the midgut tissue at wandering.

**DISCUSSION**

Although the manner of programmed cell death of the larval midgut has yet to be clearly established, it is known that mitochondria play a key role in type I (apoptosis) and type II (autophagy) programmed cell death (9, 34). Even as it is dying, the larval midgut must maintain a degree of structural and functional integrity until the pupal epithelium is formed. For this to occur, ATP production must be maintained, and, in this obligatorily aerobic tissue, this means maintaining mitochondrial function. Nevertheless, aerobic metabolism progressively degrades as metamorphosis proceeds. One day before wandering, the midgut’s respiration rate falls, and isolated midgut

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Cytochrome c, nmol/mg protein</th>
<th>Cytochrome aa₃, nmol/mg protein</th>
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<tbody>
<tr>
<td></td>
<td>Mitochondria</td>
<td>Tissue</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.25±0.02</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.20±0.01*</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.17±0.01*</td>
<td>0.05±0.002*†</td>
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Values are means ± SE for 5 different mitochondrial preparations. *Significantly different from day 2. †Significantly different from day 4.
mitochondria have a diminished capacity to oxidize succinate, although oxidation of palmitoyl carnitine is unimpaired (16). Coincident with wandering is an 80% decline in epithelial ion transport and further diminution of mitochondrial substrate oxidation (16). These findings have been confirmed in the present study, because rates of succinate oxidation are lower in midgut mitochondria isolated from *day 4* and *day 5* (wandering) larvae than from *day 2* larvae. In state 4, decreased respiration could be due to decreased activity in the substrate oxidation system and/or a decreased proton conductance of the inner mitochondrial membrane. Elasticity analysis revealed that the depressed state 4 rate seen in *day 4* and *day 5* mitochondria was not due to decreased proton permeability of the membrane but, rather, decreased activity of the substrate oxidation system. Although elasticity analysis does not identify the site(s) within the substrate oxidation subsystem that is(are) responsible for the decreased activity of the system, changes in the citric acid cycle may be important, because previous studies demonstrated that midgut mitochondrial citrate synthase activities fall between *day 2* and *day 5* (14).

The results of the present study show that the electron transport chain may be another site of modulation during metamorphosis. Mitochondria isolated from the midguts of postcommitment larvae have lower levels of cytochrome *c*, but, interestingly, it appears that the total concentration of cytochrome *c* is the same in the midguts of *day 4* and *day 2* larvae. These observations are consistent with the release of cytochrome *c* into the cytoplasm on or before *day 4*. The appearance of cytochrome *c* in the cytoplasm is an early event in many (28), but not all (27), cells undergoing programmed cell death. Loss of cytochrome *c* can lead to a decreased mitochondrial respiration rate, and, in some cases, reintroduction of cytochrome *c* can restore the respiration rate in mitochondria that have lost this protein (1, 20, 39). This restoration, however, appears to be effective only in the very early stages of cell death, and, as cell death proceeds, irreversible mitochondrial dysfunction is apparent (39, 45). Although it is not known whether midgut mitochondria can be rescued with the addition of exogenous cytochrome *c*, such a rescue might be unlikely in *day 5* mitochondria, where there is a loss of cytochrome aa3. Additional studies are needed to determine whether this loss of cytochrome *c* oxidase, which is normally found at excess capacity in mitochondria (25), is large enough to impair mitochondrial respiration when exogenous cytochrome *c* is provided.

Although the activity of the substrate oxidation system declined after commitment to pupation, this subsystem conferred ~90% of the control over state 3 respiration at all stages of development. This high level of control over respiration differs from that observed in mammalian mitochondria (29, 46) but is similar to that of plant mitochondria (33). The relatively low degree of control over state 3 respiration that is exerted by the phosphorylation system may account for the observation that the oxygen consumption of the intact midgut is apparently insensitive to the phosphorylation status of the cell. That is, inhibition of active ion transport, which should alter the ADP availability and the activity of the phosphorylation system, has no effect on mitochondrial respiration in intact midgut cells (36). In contrast, modulating the substrate oxidation system by presenting the midgut tissue with different metabolic substrates does elevate the tissue’s respiration rate (36).

The proton conductance of the inner mitochondrial membrane increases with membrane potential; therefore, comparisons of proton leak rates or proton conductances among treatment groups or species must be done at the same membrane potential. In addition, such comparisons must be done at the same temperature. The present study is the first to report proton conductances in insect mitochondria, but the value at 160 mV (−0.3 nmol H⁺·min⁻¹·mg protein⁻¹·mV⁻¹) is similar to that of frog muscle mitochondria (0.26 nmol H⁺·min⁻¹·mg protein⁻¹·mV⁻¹) measured at 25°C and 158.9 mV (49). Although the maximal state 4 membrane potential was lower in *day 5* than in *day 2* mitochondria, the proton conductance was higher. Proton permeability is affected by membrane area (43), lipid composition (32), and the presence and types of uncoupling proteins (50), but it is not known whether any of these factors change during tobacco hornworm development.

The proton leak confers about half the control over state 4 respiration, but this declines to near zero in state 3. The proton leak also confers little control over the phosphorylation system. In contrast, the phosphorylation system confers a substantial negative control over the proton leak. These patterns are similar to those observed in mammalian (22, 29, 30, 46) and plant (33) mitochondria.

In summary, the substrate oxidation system confers substantial control over oxidative phosphorylation in midgut mitochondria, and it is activity of this subsystem that declines during the early stages of metamorphosis. The large decline in the kinetics of the substrate oxidation system is reminiscent of those changes seen during metabolic depression in other organisms. Studies on hibernating ground squirrels (2), hibernating frogs (49), and estivating snails (3) show a decrease in the activity of the substrate oxidation system with no change in the proton leak. Whether the specific sites within the substrate oxidation system that are targeted during programmed cell death are the same as those targeted during metabolic depression remains to be determined.

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**GRANTS**

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