Top-down control analysis of the effect of temperature on ectotherm oxidative phosphorylation

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Chamberlin, M. E. Top-down control analysis of the effect of temperature on ectotherm oxidative phosphorylation. Am J Physiol Regul Integr Comp 287: R794–R800, 2004. First published June 10, 2004; 10.1152/ajpregu.00240.2004.—Top-down control and elasticity analysis was conducted on mitochondria isolated from the midgut of the tobacco hornworm (Manduca sexta) to assess how temperature affects oxidative phosphorylation in a eurythermic ectotherm. Oxygen consumption and protonmotive force (measured as membrane potential in the presence of nigericin) were monitored at 15, 25, and 35°C. State 4 respiration displayed a Q10 of 2.4–2.7 when measured over two temperature ranges (15–25°C and 25–35°C). In state 3, the Q10 for respiration were 2.0 and 1.7 for the lower and higher temperature ranges, respectively. The kinetic responses (oxygen consumption) of the substrate oxidation system, proton leak, and phosphorylation system increased as temperature rose, although the proton leak and substrate oxidation system showed the greatest thermal sensitivity. Whereas there were temperature-induced changes in the activities of the oxidative phosphorylation subsystems, there was no change in the state 4 membrane potential and little change in the state 3 membrane potential. Top-down control analysis revealed that control over respiration did not change with temperature. In state 4, control of respiration was shared nearly equally by the proton leak and the substrate oxidation system, whereas in state 3 the substrate oxidation system exerted over 90% of the control over respiration. The proton leak and phosphorylation system account for <10% of the temperature-induced change in the state 3 respiration rate. Therefore, when the temperature is changed, the state 3 respiration rate is altered primarily because of temperature’s effect on the substrate oxidation system.

Manduca sexta; elasticity analysis; midgut; proton conductance; Q10

MANY ECTOTHERMS EXPERIENCE ACUTE CHANGES IN ENVIRONMENTAL TEMPERATURE RESULTING IN RADICAL CHANGES IN THEIR RATE OF OXYGEN CONSUMPTION. AS A CONSEQUENCE, THERE HAS BEEN A GREAT DEAL OF INTEREST REGARDING THE EFFECTS OF ACUTE TEMPERATURE CHANGES ON ECTOTHERM MITOCHONDRIAL METABOLISM. MANY STUDIES ON BOTH VERTEBRATE (E.G., SEE Refs. 4, 5, 12, 20, 22, 29, 36, AND 37) AND INVERTEBRATE (E.G., SEE Refs. 1, 14–16, 23, AND 38) MITOCHONDRIA HAVE DEMONSTRATED THAT OVER THE PHYSIOLOGICAL TEMPERATURE RANGE OF A GIVEN SPECIES, INCREASING THE TEMPERATURE INCREASES THE RESPIRATION RATE OF ISOLATED MITOCHONDRIA. IN ADDITION, THE KINETICS OF INDIVIDUAL MITOCHONDRIAL ENZYMES (E.G., SEE Refs. 5, 14, 15, 22, 31, AND 36) ARE ALTERED BY TEMPERATURE. IT HAS BEEN DIFFICULT, HOWEVER, TO ESTABLISH TO WHAT DEGREE EACH REACTION IS RESPONSIBLE FOR THE TEMPERATURE-INDUCED CHANGES IN MITOCHONDRIAL RESPIRATION. THIS IS BECAUSE THE CONTROL OF OXIDATIVE PHOSPHORYLATION IS COMPLEX AND DISTRIBUTED (26, 30, 32), AND EACH REACTION WITHIN OXIDATIVE PHOSPHORYLATION MAY DISPLAY DIFFERENT THERMAL SENSITIVITIES. A TRAJECTABLE APPROACH FOR DEALING WITH THIS COMPLEXITY IS TOP-DOWN CONTROL ANALYSIS IN WHICH OXIDATIVE PHOSPHORYLATION IS REDUCED DOWN TO JUST THREE BLOCKS OF REACTIONS, OR SUBSYSTEMS, THAT ARE LINKED BY A SINGLE, COMMON INTERMEDIATE, THE PROTONMOTIVE FORCE. THE “SUBSTATE OXIDATION SYSTEM” ENCOMPASSES ALL THE PROCESSES (E.G., TRICARBOXYLIC ACID CYCLE, ELECTRON TRANSPORT CHAIN, METABOLITE TRANSPORTERS) THAT PRODUCE THE PROTONMOTIVE FORCE, WHEREAS THE OTHER TWO SUBSYSTEMS, “PROTON LEAK” AND “PHOSPHORYLATION SYSTEM” (E.G., F1F0-ATP SYNTHASE, ADENINE NUCLEOTIDE TRANSLOCASE), DISSIPATE THE PROTONMOTIVE FORCE.

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ranging from 14 to 42°C (27). In addition, the oxygen consumption of larvae raised from eggs obtained from the desert (11) or laboratory colonies (34) has similar sensitivities to ambient temperature. Therefore, *M. sexta* is an appropriate model organism for studying the effects of temperature on ectotherm oxidative phosphorylation.

**MATERIALS AND METHODS**

*Insects. 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 that were ~60 h beyond the molt to the fifth instar and weighed between 3 and 6.5 g were used in the present study.

*Isolation of mitochondria.* Mitochondria were isolated from 14 to 20 whole midguts as described by Chamberlin (13). The final mitochondrial pellet was suspended in isolation medium (18), and the protein content was measured (7) and adjusted to 10 mg/ml.

*Measurement of mitochondrial oxygen consumption and membrane potential.* Mitochondrial oxygen consumption and protonotive force were measured simultaneously as described by Chamberlin (13). Briefly, this entailed the dilution of mitochondria (final concentration 0.975 mg/ml) in reaction medium (13), which contained nigericin. Therefore, the mitochondrial pH gradient was collapsed, and the protonotive force was expressed entirely as membrane potential. The membrane potential was measured with a methyltriphenylphosphonium (TPMP+) electrode, and oxygen consumption was measured with a miniature Clarke-type oxygen electrode (Instech Laboratories; Plymouth Meeting, PA). Mitochondrial matrix volume and nonspecific binding were determined as previously described (13). Neither binding nor matrix volume were affected by temperature (data not shown). With the use of extra- and intramitochondrial TPMP+ concentrations, the membrane potential was calculated using the Nernst equation.

**Kinetic responses of the subsystems.** The kinetic responses of all systems were determined in the presence of 0.5 mM ADP, a concentration of ADP sufficient to achieve maximal state 3 rates at all three temperatures tested. The kinetic response of the proton leak was determined in the presence of 2.8 μg/ml oligomycin to inhibit ATP production. Small doses (1.4–9.0 mM for the 15°C studies; 0.7–7.6 mM for the 25 and 35°C studies) of malonate were sequentially added, resulting in the inhibition of oxygen consumption and depolarization of the membrane potential. The kinetic response of the substrate oxidation system was determined by sequential additions of FCCP (0.3–1.7 μM) in the presence of oligomycin. These maneuvers resulted in a stimulation of oxygen consumption as the mitochondria depolarized. Titration of the phosphorylation system was performed in the presence of hexokinase (10 U/ml) to maintain a constant state 3 rate. Sequential additions of malonate (0.2–1.7 mM for the 15°C studies; 0.3–3.1 mM for the 25 and 35°C studies) were then added resulting in a depression of oxygen consumption and depolarization of the mitochondria. 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.

**Calculations and statistics.** The data from the kinetic analyses were fitted by second- or 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. (9) and Hafner et al. (21). With the use of the mean control coefficients and elasticities, the partial integrated response coefficients were calculated as described by Ainscow and Brand (2). Partial integrated response coefficients describe how much of the temperature-induced change in mitochondrial respiration is caused by changes in each subsystem.

**Differences between oxygen consumption or membrane potential measured at different temperatures were analyzed using repeated-measures ANOVA followed by a Tukey-Kramer multiple-comparison test. Q10 s calculated from measured respiration rates were log transformed before such analysis. In all instances, *P* < 0.05 was considered to represent a significant difference.**

**RESULTS**

*Characteristics of state 4 and state 3.* Increasing temperature significantly increased the state 4 (Fig. 1A) and state 3 (Fig. 1B)
rate of respiration. In contrast, temperature had no effect on the state 4 membrane potential (Fig. 1C). The state 3 membrane potential at 35°C was slightly, but significantly, higher than that at 15°C (Fig. 1D). The Q₁₀ of state 4 respiration ranged between 2.3 and 2.7 and was significantly higher than that of state 3 or uncoupled respiration when determined between 25 and 35°C. Under state 3 and state 4 conditions, the Q₁₀ was significantly different when determined between 15 and 25° versus between 25 and 35°C (Fig. 2).

**Kinetic responses of subsystems.** Figure 3 shows the kinetic responses of the three subsystems to the membrane potential. Increasing temperature increased the rate of oxygen consumption needed to match the rate of proton leak at all membrane potentials (Fig. 3A). This is also seen when the proton leak was calculated at common membrane potentials (Fig. 4A). The Q₁₀ of the proton leak ranges between 2.8 and 3.5 when determined between 15 and 25°C and between 3.6 and 4.7 when calculated between 25 and 35°C. There was a tendency for the Q₁₀ values to fall as the membrane potential increased (Fig. 4A).

The maximal rate of uncoupled respiration was highest at 35°C (152.3 ± 5.8 nmol O₂·min⁻¹·mg protein⁻¹) compared with those measured at 25°C (79.8 ± 4.1 nmol O₂·min⁻¹·mg protein⁻¹) and 15°C (38.3 ± 2.9 nmol O₂·min⁻¹·mg protein⁻¹; Fig. 3B). In addition, the data shown in Figs. 3B and 4B clearly indicate that at any membrane potential, the flux through the substrate oxidation system increases as temperature increases. The Q₁₀ of the proton leak, however, were lower than those for the proton leak (Fig. 4).

The phosphorylation system appeared to be less sensitive to temperature than the other two subsystems (Fig. 3C). The highest state 3 potential achieved at 15°C was 158 mV. When the flux through this system was calculated at this potential and at 25°C (36.2 ± 9.0 nmol O₂·min⁻¹·mg protein⁻¹; mean ± 95% confidence limits), the value was similar to that measured at 15°C (44.2 ± 2.0 nmol O₂·min⁻¹·mg protein⁻¹; mean ± SE). At 35°C, however, the calculated value at 158 mV was higher (72.9 ± 17.6 nmol O₂·min⁻¹·mg protein⁻¹; mean ± 95% confidence limits), with an estimated Q₁₀ of 1.3.

**Flux control coefficients.** Figure 5 shows the control over respiration at all three temperatures. In state 4, the control over respiration was shared between the proton leak and substrate oxidation system and the pattern of control did not change with temperature (Fig. 5A). At 15°C, the state 3 proton leak could not be measured at the state 3 potential (see Fig. 3). Therefore, state 3 flux control coefficients at 15°C were calculated assuming that oxidative phosphorylation was an unbranched pathway with the substrate oxidation system creating the protonotive force and only the phosphorylation system dissipating it. In state 3, the substrate oxidation system conferred most of the control over oxygen consumption, and this did not change with temperature (Fig. 5B). The substrate oxidation system also conferred most of the control over the flux through the phosphorylation system (Fig. 6A). Control over the state 3 proton

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**Fig. 2.** Q₁₀₅ of mitochondrial respiration. Values are means ± SE for 5 different preparations. *Significant difference from the state 4 value measured at 25–35°C. †significant difference between the Q₁₀₅ measured at 15–25°C vs. 25–35°C.

**Fig. 3.** Kinetics of the subsystems of oxidative phosphorylation measured at three different temperatures. A: proton leak. B: substrate oxidation system. C: phosphorylation system. Values are means ± SE for 5 mitochondrial preparations. Error bars have been deleted if they were smaller than the symbol.
leak was only calculated at 25 and 35°C, and the proton leak exerted substantial control over this subsystem (Fig. 6B). The pattern of control over both the phosphorylation system and the proton leak did not change with temperature.

Partial integrated response coefficients. The substrate oxidation system was responsible for 61.5% of the increase in state 4 respiration between 15 and 25°C. Between 25 and 35°C, the substrate oxidation was responsible for 45.9% of the increase in state 4. In state 3, the substrate oxidation system was responsible for over 90% of the increase in the respiration rate as the temperature increased (Table 1).

DISCUSSION

Elasticity analysis provides a convenient approach for assessing whether an external effector modulates components of the system under study. Examination of the kinetic responses of the oxidative phosphorylation subsystems at a given membrane potential reveals that temperature has the greatest effect on the substrate oxidation system and the proton leak, whereas the phosphorylation system appears less temperature sensitive. The nature of the basal proton leak is still unresolved (8, 26), but the large Q10 observed in the present study, as well as one on reptilian mitochondria (25), point to an activated process and not simple diffusion. An increase in temperature has been shown to increase the fluidity (decrease the order) of mitochondrial membranes (14, 28), and this could change the activation energy for processes governing the leak. There have been, however, no studies on mitochondrial membranes to show that a thermally induced change in fluidity causes a change in proton flux across the membrane. As has been shown in studies on other mitochondria, the proton leak current increases in a nonlinear fashion as the membrane potential increases (“nonohmic” proton leak). This indicates there is an increase in proton conductance at higher membrane potentials, although slip of the electron transport chain (change in the stoichiometry between oxygen consumption and proton pumping) at high potentials has also been offered to explain this phenomenon (reviewed in Ref. 26). When the Q10 of the proton leak was calculated at different potentials, there was a trend for the Q10 to fall (especially between 25 and 35°C) as the potential rose. This observation points to a role for leak pathways that are both temperature and potential sensitive such that the activation energies are lower at high potentials. It is difficult, however, to speculate on what these processes might be that the mechanisms underlying the basal proton leak have yet to be clearly identified.

Assuming a H+/-O2 ratio of 12 for mitochondria oxidizing succinate, it is possible to calculate and compare the proton

![Fig. 4. Kinetics of the proton leak and the substrate oxidation system at different membrane potentials and temperatures. A: proton leak. B: substrate oxidation system. Values for respiration rates were calculated from regression equations generated for each mitochondrial preparation (n = 5) and then averaged. The Q10s were calculated for temperature ranges of 15–25°C (open bars) and 25–35°C (shaded bars).](http://ajpregu.physiology.org/)

![Fig. 5. Control of mitochondrial respiration exerted by the three subsystems of oxidative phosphorylation. A: state 4 respiration. B: state 3 respiration. Flux control coefficients were calculated for each mitochondrial preparation (n = 5) and then averaged.](http://ajpregu.physiology.org/)
M. sexta mitochondria at 25°C have a lower proton conductance when measured at 25°C (1.03 nmol H⁺·min⁻¹·mg protein⁻¹·mV⁻¹). These comparisons indicate that the kinetic effects of higher body temperature may be offset by a membrane of lower proton permeability. The net result would be to limit the proton leak rate to maintain a high level of efficiency (fraction of the protonmotive force used for ATP synthesis) of oxidative phosphorylation. Nevertheless, this proposed thermal compensation remains only speculative until studies are conducted on mitochondria isolated from the same tissue in either taxonomically related species adapted to different temperatures or the same species acclimated to different temperatures.

The present study demonstrates that control of the state 4 respiration rate is controlled by both the proton leak and substrate oxidation system. Studies on the thermal responses of mitochondria isolated from other animals have demonstrated an increase in state 4 respiration with an increase in temperature (5, 22, 23, 37). This observation is sometimes interpreted as equivalent to an increase in the proton leak (e.g., see Ref. 22), but such an interpretation cannot be made accurately without measuring the membrane potential. If the proton leak were the only process to change with temperature, then the state 4 membrane potential would change. The data in the present study clearly demonstrate that this is not the case. Therefore, the activities of both the proton leak and substrate oxidation system are similarly affected by temperature. In addition, the substrate oxidation system exerts a great deal of control over state 4 respiration rate. As a result, only 38.5% of the change in state 4 respiration between 15 and 25°C is due to temperature’s effect on the proton leak. Between 25 and 35°C, the proton leak is responsible for 54.1% of the temperature effect on state 4.

At the maximal state 3 rate, the substrate oxidation system exerted over 90% of the control over the respiration rate. This is in agreement with an earlier study on tobacco hornworm midgut mitochondria (13). Calculation of the partial integrated response coefficients reveals that over 90% of the temperature-induced increase in state 3 respiration was due to temperature’s effect on the substrate oxidation system. This is not surprising given that the partial integrated response coefficient takes into account both the control over respiration rate as well as the change in flux caused by the external effector, temperature (2). Despite the fact that temperature affected the kinetics of all of the subsystems, control over respiration did not change.

Although the substrate oxidation system confers most of the control over the maximal state 3 respiration rate and is responsible for the changes in membrane potential at different temperatures, Table 1 demonstrates that the control of state 4 respiration is exerted over 90% of the control over the respiration rate. This is in agreement with an earlier study on tobacco hornworm midgut mitochondria (13). Calculation of the partial integrated response coefficients reveals that over 90% of the temperature-induced increase in state 3 respiration was due to temperature’s effect on the substrate oxidation system. This is not surprising given that the partial integrated response coefficient takes into account both the control over respiration rate as well as the change in flux caused by the external effector, temperature (2). Despite the fact that temperature affected the kinetics of all of the subsystems, control over respiration did not change.

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<table>
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<tr>
<th>Temperature Range</th>
<th>Proton Leak</th>
<th>Substrate Oxidation System</th>
<th>Phosphorylation System</th>
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Calculations are described in the text. Partial integrated response coefficients were scaled to a sum of 1.
sible for over 90% of the temperature-sensitive increase in respiration, the present study cannot identify what individual reaction(s) within the subsystem confer(s) the temperature-sensitive control over the respiration rate. Future studies must be designed and conducted to see how metabolite transporters, tricarboxylic acid cycle reactions, and/or the electron transport chain contribute to this system’s control over *M. sexta* mitochondrial respiration. In other ectotherms (5, 14), however, one component of this subsystem, cytochrome *c* oxidase, has a thermal response that is similar to that of mitochondrial respiration. These observations indicate that the thermal response of this enzyme may be responsible, in part, for the temperature-induced changes in mitochondrial respiration. Blier and Lemieux (5), however, concluded that thermally-induced changes in trout cytochrome *c* oxidase activity would have little effect on the respiration rate because the enzyme’s activity is in excess of that needed to maintain the mitochondrial respiration rate. While it is true that cytochrome *c* oxidase is generally found in excess capacity in mitochondria (19), it can confer as much as 20% of the control over mitochondrial respiration (35). Therefore, thermal effects on this enzyme may affect the respiratory rate. Nevertheless, the extent temperature affects the control exerted by this enzyme over the mitochondrial respiration of ectotherms, in general, or *M. sexta*, in particular, has yet to be determined.

In conclusion, it is not at all surprising that changes in temperature affected the activities of the subsystems of oxidative phosphorylation, but it is important to note what did not change with temperature. The maximal state 4 and state 3 membrane potential changed little or not at all over the 20°C challenge imposed in the present study. Although temperature affected the flux through all three subsystems, the constancy of the maximal state 4 and state 3 membrane potentials indicates that temperature equally affected the processes that create and dissipate the protonmotive force. Maintenance of the protonmotive force over a wide range of temperatures ensures a constant driving force for a variety of processes such as metabolite transport, ion transport, and ATP production (26, 33).

The control over respiration also did not change with temperature, despite the thermally induced change in flux. It is unlikely that this thermally stable control pattern is an adaptation to fluctuating body temperatures because the control of mammalian mitochondrial respiration is similarly unaffected by changes when the temperature is decreased from 37 to 25°C (17). Instead, this constancy may be an intrinsic property of animal oxidative phosphorylation.

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