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 an 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 accounted 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 tractable 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 proton mototive force. The “substrate oxidation system” encompasses all the processes (e.g., tricarboxylic acid cycle, electron transport chain, metabolite transporters) that produce the proton mototive force, whereas the other two subsystems, “proton leak” and “phosphorylation system” (e.g., F1F0-ATP synthase, adenine nucleotide translocase) dissipate the proton mototive force. Because the blocks share a common intermediate, perturbation of one block will affect the proton mototive force and thus alter flux through the other block(s).

The kinetic response (oxygen consumption) of each block to changes in the proton mototive force is determined, and the elasticities (fractional change in oxygen consumption divided by the fractional change in the proton mototive force) of each subsystem can then be used to calculate flux control coefficients (21). Although such an approach does not quantify the control exerted by individual reactions, this technique does provide a way to quantify the control that each subsystem confers over the system. Therefore, a broad view of how temperature affects the control of oxidative phosphorylation is obtained.

The same data used to calculate control coefficients can be used for elasticity analysis, which allows a comparison of how each subsystem is affected by an external effector such as temperature. Dufour and co-workers (17) studied rat liver mitochondria at 37, 25, and 4°C. Although elasticity analysis revealed that the activities of all subsystems were affected by temperature, the magnitude of control exerted over state 3 respiration by the individual subsystems was the same at 37 and 25°C. At 4°C, however, the phosphorylation system increased control over the state 3 respiration rate. To determine whether a similar pattern of control occurs in mitochondria from an animal that normally experiences wide swings in body temperature, the present study employed top-down control and elasticity analysis to study the effect of temperature on mitochondria isolated from the midgut of the tobacco hornworm (Manduca sexta). This lepidopteran species has a wide geographic distribution throughout North, Central, and South America (24) and grows at environmental temperatures ranging from ~10°C (6) to over 35°C (3, 10, 39). In addition, daily fluctuations in ambient temperature can lead to changes in body temperature of at least 20°C (3, 10, 39). Although most physiological studies of this species (including the present study) have been performed on laboratory stock raised for generations at temperatures around 25–27°C, these domesticated insects feed and grow while exposed to temperatures ranging from 10–35°C (6) to over 35°C (3, 10, 39). Therefore, a broad view of how temperature affects the control of oxidative phosphorylation is obtained.
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 protonmotive 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 protonmotive 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 (Intech 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 Hafer 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 values 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°C 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 Q10s, 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 protonmotive 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. Q10s 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 Q10s measured at 15–25°C vs. 25–35°C.
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 (“non-ohmic” 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 given that the mechanisms underlying the basal proton leak have yet to be clearly identified.

Assuming a H+/O ratio of 12 for mitochondria oxidizing succinate, it is possible to calculate and compare the proton flux control coefficients were calculated 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).

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).

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.
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Fig. 6. Control of the phosphorylation system and proton leak exerted by the three subsystems of oxidative phosphorylation. A: phosphorylation system. B: proton leak. Flux control coefficients were calculated for each mitochondrial preparation (n = 5) and then averaged.

Table 1. Partial integrated response coefficients

<table>
<thead>
<tr>
<th>Temperature Range</th>
<th>Proton Leak</th>
<th>Substrate Oxidation System</th>
<th>Phosphorylation System</th>
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<tbody>
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<td>15–25°C</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>State 4</td>
<td>0.385</td>
<td>0.615</td>
<td>0.004</td>
</tr>
<tr>
<td>State 3</td>
<td>0.000</td>
<td>0.996</td>
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<tr>
<td>25–35°C</td>
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<td></td>
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<tr>
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<tr>
<td>State 3</td>
<td>0.010</td>
<td>0.915</td>
<td>0.075</td>
</tr>
</tbody>
</table>

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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