Supplementary information

Global metabolite profiles under hypoxia

Metabolite concentrations were measured at 10 minutes, 1 hour, and 4 hours to study the time course of adaptation and breakdown of the metabolic network under hypoxic stress. A comparison of Figure 1S with a previous study of hypoxic isolated mouse cells (Troy, 2005) reveals obvious differences between insect and mammalian metabolic makeup and response to hypoxia. Changes in lactate, alanine, and acetate peaks can be seen clearly (boxed in red), compared with the relative stability of the size and shape of the background spectra. Water suppression techniques were successful for 22 of the 25 spectra, and in the remaining three the water signal masked some peak clusters of trehalose and glucose.

The spectra of some metabolites overlap considerably, which decreases confidence when assigning concentrations to those compounds. As a result, relative changes are measured more precisely than absolute concentrations, and certain metabolites were quantified with more certainty than others. Figure 2S shows the complete results of metabolite quantification, separated into high and low confidence groups according to amount of peak overlap. The results of metabolite quantification show that many compounds remained at a constant level for all time points. Notably, of the metabolites quantified, none are seen to decrease. However, this is not necessarily an artifact. An increase in total NMR spectrum does not betray conservation of mass, since these additional small molecules may be the breakdown products of the proteins, membranes, and other macromolecules that are normally discarded during sample preparation. Also, trehalose and glycogen, the predominant sources of anaerobic energy, were not measured. Although glycogen peaks can be seen in the spectra, the absolute concentration cannot be quantified from the NMR spectra due to the fact that there are a variable number of hydrogen atoms in each glycogen polysaccharide. Trehalose has a high binding affinity to proteins, which are filtered from the sample before measurement.

Additional enzymatic assays will be necessary to quantify the true time course of trehalose and glycogen concentrations. Variability in glycerol concentrations may be a result of residual glycerol on the filters used in sample preparation. Additional assays must also be done to determine true changes in glycerol during hypoxia.

In the absence of oxygen it can be assumed that only accumulation occurs for certain end products, and only depletion occurs for certain substrates. For example, lactate is a product of the single enzyme lactate dehydrogenase (LDH), and under extreme hypoxic conditions this reaction is irreversible. Therefore, the time course of lactate concentrations can be assumed to closely follow LDH flux. Drosophila heart rate slows drastically under hypoxic conditions (J Feala, unpublished data, 2006), and the resulting reduced circulation would prevent these byproducts from exiting the system and escaping measurement. Similar assumptions can be made for the end products acetate and alanine, and for the depletion of the common flight muscle substrates glycogen, trehalose, and proline. Glycogen and trehalose were not measured by NMR, and will be assayed by biochemical methods in the future. Proline concentrations did not change significantly during hypoxia. Although this substrate is required for flight, proline oxidation pathways were not considered to be important for hypoxic ATP generation.
Acetate, alanine, and lactate showed a statistically significant increasing trend with a good linear fit, but the actual time course more closely represented an exponential rise to saturation (Figure 3S). However, since flux balance analysis rests on the assumption of steady state, reaction fluxes were derived from the slope of the linear approximation. Figure 3S displays the three trendlines, and Table 1 lists the fluxes and \( R^2 \) goodness-of-fit values.

**Model validation**

The core energy-producing pathways in the model, consisting of glycolysis, TCA (tricarboxylic acid) cycle, electron transport chain, and oxidative phosphorylation, were validated using experimentally measured ratios of ATP produced per molecule of glucose and ATP per oxygen atom (P/O or ADP/O ratio).

Representative oxygen electrode recordings from a study of isolated *Drosophila* mitochondria (Ferguson et al., 2005) were used to approximate the P/O ratio in flight muscle using pyruvate/proline and glycerol-3-phosphate as NAD- and FAD- linked substrates, respectively. These conditions were simulated in the model by constraining cytosolic pathways to zero while adding a flux of ADP in the presence of an infinite amount of substrate. The rate of oxygen consumption was compared directly to the linear portion of state 3 respiration in the experimental oxygen traces. The stoichiometry of electron transport within the model was refined to better approximate experimental results.

Whole cell simulations were performed to calculate the amount of ATP produced per glucose, with no other carbon source added. Since measurements of ATP:glucose ratio were not found in the literature, the results were compared with ATP:glucose ratios in human mitochondria.

The ADP/O ratio produced by simulation was compared to that of experiment by constraining the ADP flux to experimental value (≈ 8.9 nmol/min*µg protein) in a saturated solution of pyruvate and proline substrate as described in Ferguson, et. al (2005). Oxygen uptake in the linear region of state 3 respiration was then approximated from the oxygen electrode trace and the ADP/O ratio was calculated using the formula: [ADP]/2[O_2]. These conditions were simulated in the model and the resulting oxygen uptake rate was compared to experiment. After noting a large discrepancy, the stoichiometry of proton transfer was adjusted for NADH dehydrogenase and cytochrome reductase, two reactions in the electron transport chain. These refinements increased the ADP/O ratio from 2.12 to 2.76 in the model, the latter value comparing more favorably with the experimental value of 3.34 (a percent error of 17% vs 36%). The FAD'-linked substrate \( \alpha \)-glycerol-3-phosphate produced a poorly defined oxygen trace; however, a rough approximation of oxygen uptake provided an ADP/O ratio of 1.33. Simulation of the isolated mitochondria in the presence of \( \alpha \)-GP produced a ratio of 1.75. The refined model produced an ATP:glucose ratio of 33.0, which is similar to the reported value in humans of 31.5 (Nelson, 2000).
Supplementary Figure Legends

**Figure 1S:** Representative NMR spectra with emphasis on accumulating end products.

**Figure 2S:** Complete metabolic profile calculated from NMR spectra. Metabolites are separated by confidence in peak alignment and integration.

**Figure 3S:** Calculating end product accumulation fluxes from time course data. These curves are better fit by an exponential rise to saturation, but flux-balance analysis requires steady state flux.

**Figure 4S:** Diagram of ATP-producing pathways included in the constraint-based model, but only including acetate and alanine pathways utilized by the simulation.

References

