Control Analysis of energy metabolism in mitochondria
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Metabolic Control Analysis
Metabolic Control Analysis was first used experimentally on oxidative phosphorylation by Groen and co-workers [1]. Since then, several different research groups have used Control Analysis in a number of different ways to gain a deeper understanding of the control of this complex process in isolated mitochondria, in intact cells, in perfused tissues and in living organisms [2-6]. As a result, oxidative phosphorylation now provides the best example of the experimental application of Metabolic Control Analysis. Our contribution to this progress has been to develop and apply a top-down, or modular, approach to Control Analysis [7-9] that greatly simplifies the experiments that are required, whilst giving powerful insights into the system behaviour of complex pathways. We have used this top-down approach to investigate the control and regulation of energy metabolism in organelles, cells and tissues.

Experimental application of Metabolic Control Analysis has tended to concentrate on the measurement of Control Coefficients. These describe and quantify the distribution of control within pathways. However, Control Analysis also encompasses several other ways of measuring control and regulation. Recently, there has been growing interest in other coefficients that describe regulation in ways that are related to those more familiar to traditional metabolic biochemists.

For example, the comparison of elasticity curves that describe the overall kinetic responses of reactions to intermediates can be used to identify the sites of action of effectors within a complex system. We have termed this approach Elasticity Analysis, and have used it extensively [5,6,10-12].

Another example of the value of other coefficients within Metabolic Control Analysis is provided by Response Coefficients, in a context that we have termed Regulation Analysis. Response Coefficients quantify the response of a system to an external effector such as an added inhibitor or hormone [13]. A Response Coefficient can be broken down to a set of Partial Response Coefficients that describe the regulatory strength of the effector acting via different steps in the pathway [14,15]. Similarly, the importance of different local effectors of a target enzyme in mediating the response of that enzyme to an external effector can be measured [16].

Response Coefficients can also be extended to describe the response of a system to the concentrations of each of its internal intermediates (Internal Response Coefficients); these in turn can be broken down to Partial Internal Response Coefficients that describe and quantify the regulatory strength of an intermediate over a pathway via each of its target enzymes [14,15].

Description of metabolic regulation using these different types of Response Coefficient corresponds much more closely to the traditional biochemical approach of attempting to describe the regulation of metabolism by describing the effects of hormones or metabolites on different enzymes. Thus a Response Coefficient to a hormone could quantify its effect on the rate of a pathway, and Partial Response Coefficients to the hormone could show how each of the primary actions of the hormone contributed to the overall effect. Similarly, Partial Internal Response Coefficients of energy metabolism to one of its intermediates, such as ATP, could quantify the importance of each of the activatory and inhibitory actions of ATP on different enzymes to the maintenance of the overall steady state in the system.

Despite its great potential, no full experimental description of the regulation of a metabolic system using Regulation Analysis has yet been published. Here we show how Metabolic Control Analysis (Elasticity Analysis, Control Analysis and Regulation Analysis) can give a quantitative description of the multiple actions of an external effector (cadmium) on a complex metabolic system (mitochondria isolated from potato tubers) [17-19].

Metabolic Control Analysis of the effects of cadmium on oxidative phosphorylation
Cadmium is a heavy metal that has significant toxicological effects in the environment [17]. We investigated its multiple effects on oxidative phosphorylation in isolated potato tuber mitochondria as a convenient model system in which to study its biological effects and to explore the insights that can be gained by using Metabolic Control Analysis [17-19]. We used a top-down approach to simplify the system to make it tractable. To do this we conceptually divided oxidative phos-
phorylation into three blocks of reactions connected by the protonmotive force ($\Delta p$) [20]. These blocks (or subsystems, or modules) comprised (i) the substrate oxidation subsystem, consisting of all the reactions of the electron transport chain that together pump protons across the mitochondrial inner membrane to form $\Delta p$, (ii) the phosphorylation subsystem, consisting of all the reactions that consume $\Delta p$ to form and then hydrolyse ATP, and (iii) the proton leak, consisting of all reactions that consume $\Delta p$ without ATP synthesis. We then carried out Elasticity Analysis, Control Analysis and Regulation Analysis to describe the effects of cadmium on this simplified four-component system. Experimentally, this involved performing a series of simple titrations of flux and $\Delta p$ using inhibitors of different blocks, at different cadmium concentrations and different rates of oxidative phosphorylation.

**Elasticity Analysis of the effects of cadmium on oxidative phosphorylation**

The first step of the overall Metabolic Control Analysis was to carry out an Elasticity Analysis to identify the subsystems that cadmium affects directly and to measure their Elasticities to $\Delta p$ and to cadmium. To do this we measured the rate of each of the three blocks as a function of $\Delta p$, which is the product of substrate oxidation and the driving force or substrate for phosphorylation and proton leak. To measure the kinetic curve for a given subsystem, $\Delta p$ was varied by titration of a different subsystem with inhibitors or activators, and the value of $\Delta p$ and the rate of the subsystem of interest in the steady state were measured [17].

The curves describing the kinetic response of the substrate oxidation subsystem to $\Delta p$ at different cadmium concentrations did not superimpose, but showed progressive stimulation of proton leak rate at any $\Delta p$ value as cadmium concentrations increased. Thus there is a primary site of activation by cadmium on the proton leak reactions.

Elasticities are defined as the fractional change in subsystem rate caused by an infinitesimal fractional change in effector concentration when all other effectors are held constant. There are two relevant types of Elasticity in these experiments: Elasticity to $\Delta p$ at fixed cadmium concentration and Elasticity to cadmium at fixed $\Delta p$. We measured these two Elasticities for each of the three subsystems under a range of conditions: different free cadmium concentrations between zero and 21 $\mu$M, and different rates of oxidative phosphorylation between resting (state 4) and maximal ATP turnover (state 3).

We calculated the Elasticities of each subsystem to $\Delta p$ under each condition with fixed cadmium concentration from the slopes of the kinetic plots of subsystem rates against $\Delta p$ at the appropriate value of $\Delta p$. These Elasticities were used to calculate Control Coefficients (see below).

We calculated the Elasticities of each subsystem to cadmium under each condition with fixed $\Delta p$ as follows. For a given condition of respiratory state and cadmium concentration, we noted $\Delta p$. From the graphs of subsystem rates against $\Delta p$ we then read off the rate of a subsystem at that value of $\Delta p$ for each of the other cadmium concentrations, then replotted these rates at fixed $\Delta p$ against cadmium concentration. We then calculated the Elasticities of each subsystem to cadmium under each condition with fixed $\Delta p$ from the slopes of these replots. These Elasticities were used to calculate Partial Response Coefficients (see below).

The Elasticities of the three subsystems to cadmium are shown in Figure 1. The substrate oxidation subsystem was inhibited by cadmium, as shown more qualitatively above by the Elasticity Analysis. It was particularly sensitive to small changes in cadmium concentration at the lower cadmium concentrations near state 4. The phosphorylation subsystem was very insensitive to cadmium, with Elasticity Coefficients near zero under all conditions. The proton leak subsystem was activated by cadmium, as shown more qualitatively above by the Elasticity Analysis. It was sensitive to cadmium under all conditions, but particularly so at higher cadmium concentrations near state 3. Plotting our Elasticities in this way shows particularly clearly exactly how the sensitivity of reactions to effectors varies with condition [21].
Elasticities ($e$) of the substrate oxidation subsystem ($S$), the phosphorylation subsystem ($P$) and the proton leak ($L$) to cadmium under different conditions

Elasticities in isolated potato tuber mitochondria were calculated from graphs of subsystem rate against cadmium concentration at fixed $\Delta \rho$ in different respiratory states set by addition of hexokinase (HK) in the presence of glucose to regenerate ADP at different rates. For details see [19]. Endo-HK, endogenous HK (none added).

Control Analysis of oxidative phosphorylation at different cadmium concentrations

The second step of the overall analysis was to perform a Control Analysis to identify the significant sites of control within the system in the presence of different concentrations of free cadmium [18]. Control Coefficients are defined as the fractional change in a system variable (such as respiration rate) caused by an infinitesimal change in subsystem activity. They can be simply and conveniently calculated from the Elasticities to $\Delta \rho$ and the subsystem rates as described elsewhere [9, 18, 20]. The data set described above allows the calculation of the control exerted by each subsystem over the rate through each subsystem, over the ratio of rates through each pair of subsystems and over the value of $\Delta \rho$; this gives a total of 21 different Control Coefficients, each of which has a different value under each of the 24 different combinations of cadmium concentration and respiratory state that we examined. This wealth of control information can be used to highlight many different aspects of the effects of cadmium on system behaviour. In Figure 2 we select one subset of the results to show how control over respiration rate is distributed between the three subsystems in different respiratory states, and how this pattern of control is generally maintained but squeezed to lower respiration rates in the presence of cadmium.

In the absence of cadmium, control over respiration rate in potato tuber mitochondria oxidizing NADH is shared equally between substrate oxidation and proton leak. As respiration rate is increased by increasing the ADP supply, control by phosphorylation becomes dominant. As the respiratory rate reaches state 3, control by phosphorylation diminishes and control by proton leak disappears.
while control by substrate oxidation becomes pre-
eminent. Two general properties of metabolic con-
trol are illustrated nicely by this experiment: control is shared between different reactions, with no single 'rate-limiting' step, and the contribution of any step to control can vary a great deal depending on the conditions.

In the presence of cadmium the pattern of control is similar, but control by substrate oxidation (which is inhibited by cadmium) is enhanced and control by the proton leak (which is stimulated by cadmium) is decreased. However, even in a simple system like this one, it is not safe to use Control Coefficients to attempt to identify sites of action of effectors: the control by phosphorylation is decreased by cadmium even though cadmium has no primary effect on the phosphorylation subsystem itself. The phosphorylation subsystem loses control as a secondary result of the changes in $\Delta \rho$ caused by the primary effects of cadmium on the substrate oxidation and proton leak subsystems. The inadequacy of changes in Control Coefficients to pinpoint primary sites of action of effectors is a general rule [18]; Elasticity Analysis should be used instead.

**Regulation Analysis of the effects of cadmium on oxidative phosphorylation**

The third step of the overall analysis was to carry out a Regulation Analysis to calculate the overall response of the system to cadmium and the Partial Response Coefficients that quantify the contribution of each site of cadmium action in producing the overall effect.

The Response Coefficients of each of the system variables to cadmium can be simply calculated from the slopes of plots of the variable (such as respiration rate) against cadmium concentration under different conditions. Figure 3 shows an example; the overall Response Coefficient of respiration rate to cadmium is plotted at each condition that was investigated. The graph shows quantitatively how cadmium inhibits the respiration rate under most conditions, but stimulates it at high cadmium concentrations near state 4.

Intuitively, we can explain the inhibition by cadmium under some conditions and the stimulation under others by the antagonistic cadmium effects of inhibition of substrate oxidation but stimulation of proton leak. The effect on the proton leak must dominate near state 4, while the effect on substrate oxidation must dominate elsewhere. The calculation of Partial Response Coefficients to cadmium allows this intuitive view to be expressed quantitatively [19].

The overall Response Coefficient of respiration rate to cadmium can be divided into a set of Partial Response Coefficients that quantify how cadmium acts on each subsystem to produce its overall effect. The effect of cadmium on respiration rate acting through a particular subsystem is the product of the Elasticity of that subsystem to cadmium and the Control Coefficient of that subsystem over respiration rate. Thus cadmium will have little effect on respiration rate through the phosphorylation subsystem even though phosphorylation is the dominant controller of respiration rate at intermediate respiration rates, since the Elasticity of phosphorylation to cadmium is always low. Similarly, cadmium will have little effect on respiration rate through the proton leak in state 3 even though the leak is very sensitive to cadmium, since the leak has little control over respiration rate under these conditions. Only when cadmium significantly affects a subsystem that exerts significant control over respiration rate will there be any effect on respiration rate via this subsystem. The overall Response Coefficient to cadmium is given by the sum of the Partial Response Coefficients, so the relative importance of the effects of cadmium on different subsystems under any defined condition can easily be obtained.

Figure 3 shows the Partial Response Coefficients of respiration rate in potato tuber mitochondria to cadmium acting through the three subsystems. Respiration is sensitive to cadmium inhibition of substrate oxidation under all conditions, particularly at low cadmium concentrations near state 4. Respiration is insensitive to cadmium acting through phosphorylation, since cadmium has little effect on this subsystem. Respiration is not very sensitive to cadmium activation of proton leak under most conditions, but becomes very sensitive near state 4.

The quantitative explanation for the overall pattern of inhibition and activation of respiration by cadmium is now clear. Cadmium tends to stimulate respiration at high cadmium concentrations near state 4 because the high Elasticity of the leak to cadmium (Figure 1) and the quite high control of the leak over respiration rate in state 4 (Figure 2) combine to give a large stimulatory effect via proton leak (Figure 3). Cadmium tends to inhibit respiration under the same conditions via its effects on the substrate oxidation subsystem (Figure 3) because substrate oxidation has quite high control over respiration rate (Figure 2) and there is a significant
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Partial Response Coefficients ($R$) of respiration rate ($J$) to cadmium exerted through substrate oxidation ($S$), phosphorylation ($P$) and proton leak ($L$) in potato tuber mitochondria under different conditions

Partial Response Coefficients were calculated by multiplying the appropriate elasticities (Figure 1) and Control Coefficients (Figure 2 and other data not shown) [19]. The overall Response Coefficients ($\ast R$) were calculated by adding the Partial Response Coefficients; direct calculation of overall Response Coefficients from graphs of respiration rate against cadmium concentration gave identical results.

Elasticity of substrate oxidation to cadmium under these conditions (Figure 1). The stimulatory effect of cadmium on respiration expressed by the Partial Response Coefficient through proton leak is greater than the inhibitory effect expressed by the Partial Response Coefficient through substrate oxidation (Figure 3), so overall there is a small stimulatory effect of cadmium at high cadmium concentrations near state 4. Under all other conditions the inhibitory effect via substrate oxidation overcomes the stimulatory effect via proton leak, since the control of the proton leak over respiration rate diminishes as respiration rates increase above state 4, and the elasticity of substrate oxidation to cadmium increases at lower cadmium concentrations.

**Conclusion**

Together, the Elasticity Analysis, Control Analysis and Regulation Analysis give a full, quantitative description at the system level of the multiple actions of cadmium on the complex pathway of oxidative phosphorylation in potato tuber mitochondria. The raw data are not difficult to generate: all that is required is measurement of $\Delta \rho$ and respi-
oration rate during simple titrations with inhibitors or activators that act exclusively within the different subsystems, such as hexokinase (that acts within the phosphorylation subsystem) and cyanide (that acts on substrate oxidation). Such a description can be a valuable tool for the quantitative investigation of the multiple effects of multi-site inhibitors on complex systems in general. It can also act as a model for the analysis of the actions of other, more physiological, effectors such as calcium and hormones on energy metabolism in mitochondria or in intact cells and organs.

Introduction

Many of our ideas about the control of flux have been based on measurements of the kinetic properties of isolated enzymes in vitro. While undoubtedly a valuable approach, it presumes a detailed knowledge of the intracellular environment of these enzymes in terms of their free substrate and effector concentrations and possible interactions with other cell proteins, which may modulate their activity.

Defining the intracellular environment of an enzyme represents a considerable challenge. For example, the concentrations of substrates and effectors available to a specific enzyme cannot simply be estimated from measurements on cell extracts if the enzyme and its substrates are partitioned between cell compartments. A more subtle form of substrate compartmentation results from the very high protein concentrations in the cell, which can reach 200-300 mg/ml in the cytosol and 600-700 mg/ml in the mitochondrial matrix [1]. Thus the extractable concentration of a metabolite may be very much higher than the kinetically relevant free form if a significant fraction of the metabolite is protein-bound in the cell. The concentrations of many of the enzymes involved in cellular energy metabolism, for example, are in the range 1-100 µM [2], which is comparable in many cases to the concentrations of their substrates in the cell [3]. Comparability between the concentrations of an enzyme and organs.


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Probing the properties of enzymes in vivo using NMR

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Introduction

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Abbreviations used: TCA, tricarboxylic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase.

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