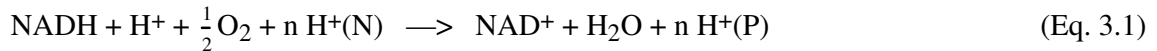


## Chapter 3 A modular approach to control analysis of respiratory chain networks

### § 3.1 The flexible topology of respiratory chain organisation

In the majority of cells oxidation of the reduced cofactor NADH is catalysed by a collection of membrane enzymes, the respiratory chain. Limiting us to aerobic respiration where molecular oxygen is the ultimate oxidant, the net reaction is:



The  $n$  protons are transported from one side of the membrane (N) to the other (P), and this transport is obligatory linked to the electron transfer. This takes care of conservation of the energy from the exergonic redox reaction in the form of a proton gradient.

The classical respiratory chain that can be admired in many biochemistry texts is that of mammalian mitochondria. It's organisation is roughly sketched in Fig. 3.1. It can be seen as a limited number (3) of enzymes, catalysing electron transfer from an input (NADH) to an output ( $\text{O}_2$ ) via two way stations, coenzyme Q (Q) and cytochrome  $c$  (cyt.  $c$ ). Additionally another input, from the citric acid cycle intermediate succinate is shown, which introduces a fourth enzyme into Fig. 3.1. There are more inputs, but as succinate and NADH-linked substrates are most commonly used in *in vitro* experiments, only these two are shown here. The intermediate compounds Q and cyt.  $c$  are thought to function as "pools", which means there is complete redox equilibration between all Q molecules, and also between all cyt.  $c$  molecules.

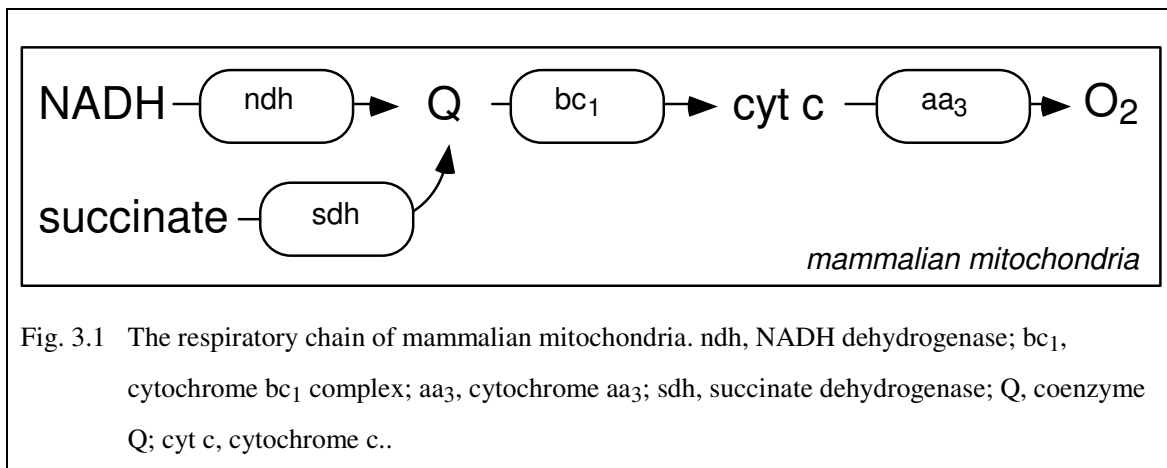


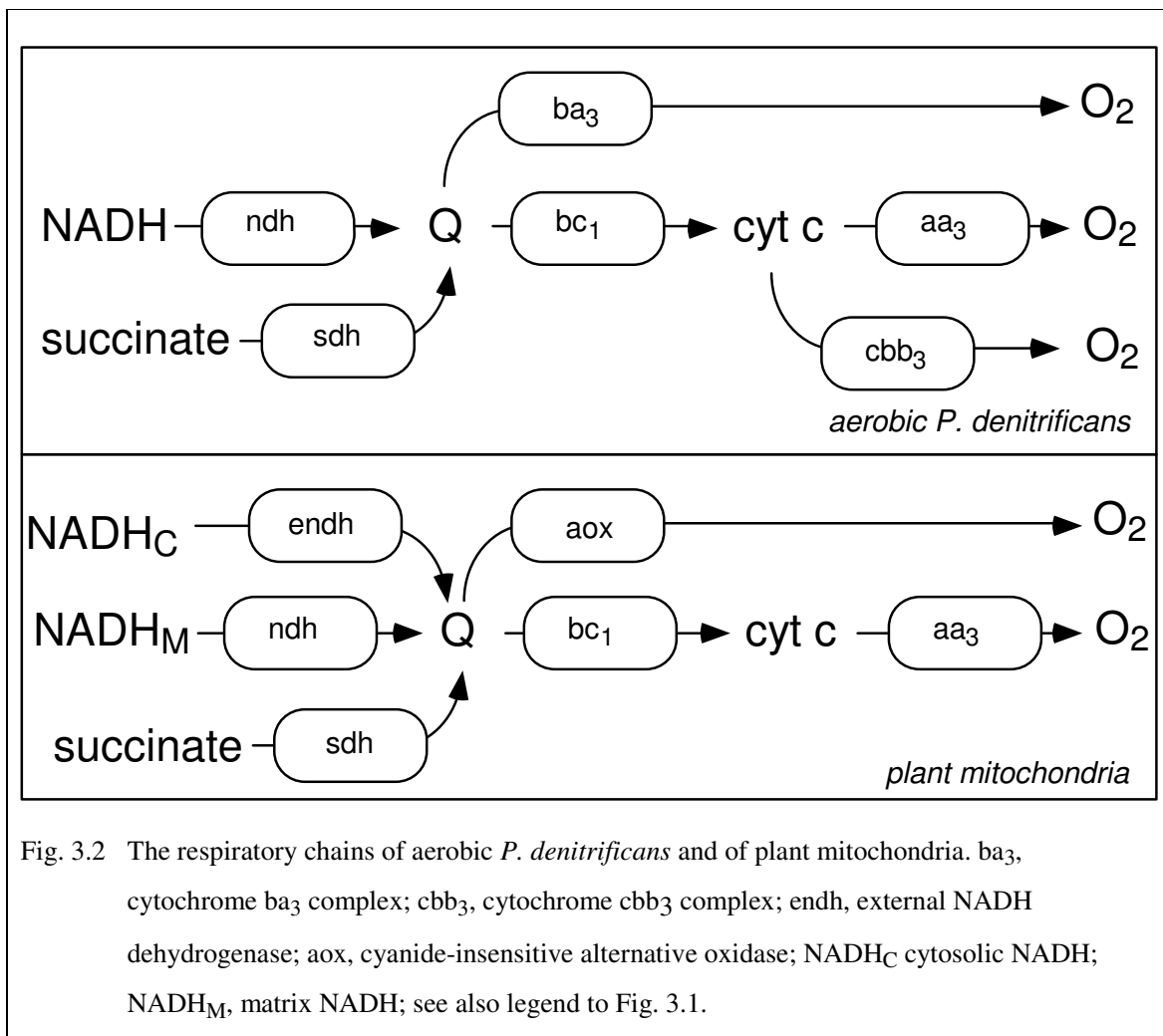
Fig. 3.1 The respiratory chain of mammalian mitochondria. ndh, NADH dehydrogenase;  $bc_1$ , cytochrome  $bc_1$  complex;  $aa_3$ , cytochrome  $aa_3$ ; sdh, succinate dehydrogenase; Q, coenzyme Q; cyt  $c$ , cytochrome  $c$ .

In a number of systems the overall scheme of respiratory flux is more complicated: in Fig. 3.2 the respiratory chains of plant mitochondria and aerobic *Paracoccus denitrificans* are shown. In these chains more pathways to oxygen are present (or, rather, possible), introducing branches that turn a simple respiratory chain into a respiratory network. In plant mitochondria also a third input from NADH in the cytoplasm (as opposed to NADH in the matrix of the mitochondria) is important, so this distinction is made in the bottom half of Fig. 3.2.

As soon as we are dealing with a network rather than a chain, and as we learn that the number of protons ( $n$  in Eq. 3.1) that are translocated per two electrons transferred depends on the path through the network that is followed, it becomes interesting how electron flux is divided between the different branches, and how this division is being regulated.

## § 3.2 A look inside by modular kinetic analysis

The distribution of electron flux through the different branches of the respiratory network depends on the kinetic properties of the enzymes that, together with the intermediate pools, form the network. Here we limit ourselves to the metabolic level, so that the composition of the network (amount of enzymes and intermediate compounds) does not change. We then can divide the factors that affect the rate of any enzyme-catalysed step in the network roughly in two classes: interaction of the catalysing enzyme with its substrates and products ("simple kinetics"), and interaction with regulating compounds both within and outside the respiratory network ("regulation of activity"). We will limit our discussion to simple kinetics, so that the only way different enzymes within the network interact is via competition for common substrates (or products).



There is a relatively simple way to determine the kinetic characteristics of the enzymes that are relevant for competition for a common substrate. This is most easily explained using the network shown in Fig. 3.3 (a simplified version of Fig. 2.1), where an input and an output enzyme both interact with an intermediate compound S. Note that both reactions (input and output) are reversible, so variation of *s* (the concentration of S) affects the rates of both reaction steps.

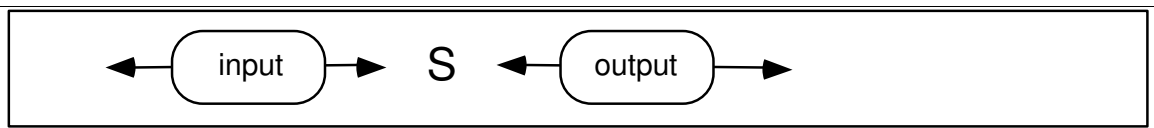


Fig. 3.3 Two-step system. S. intermediate compound produced by reversible input and consumed by reversible output.

When this system functions under conditions where the rates only vary as a function of  $s$ , the output rate obviously increases when  $s$  increases, and the input rate decreases when  $s$  increases (because of the reversible, product-sensitive nature of the input reaction). In practice, this means that a steady state will be reached in which input and output rates are both equal to the throughput flux,  $J$  of the system. Evidently, in this steady state  $s$  will have a constant value.

The steady-state values of  $s$  and  $J$  are determined by the actual dependencies of the input- and output rates on  $s$ . This gives us a tool to determine these dependencies. When the input enzyme is partially inhibited, the concentration of  $S$  and the flux  $J$  are lowered to a new steady state. However, nothing changes in the dependence of the output enzyme on  $s$ . So each successive steady state when the input is gradually inhibited yields values of  $s$  and  $J$  that reflect the dependency of the output rate on  $s$ . So inhibition of the input module yields the kinetics of the output module. In a mirror image experiment, gradual inhibition of the output (which raises  $s$  and lowers  $J$ !) gives the kinetics of the input module. This is illustrated in Fig. 3.4. This way to extract kinetic information from the system is "modular kinetic analysis". Note that this approach only is possible when we have the means to modulate input and output independently. Higher rates can be achieved by stimulating input or output rates, or by introducing extra input or output pathways. All is allowed, as long as the "following" module is only affected via  $S$ .

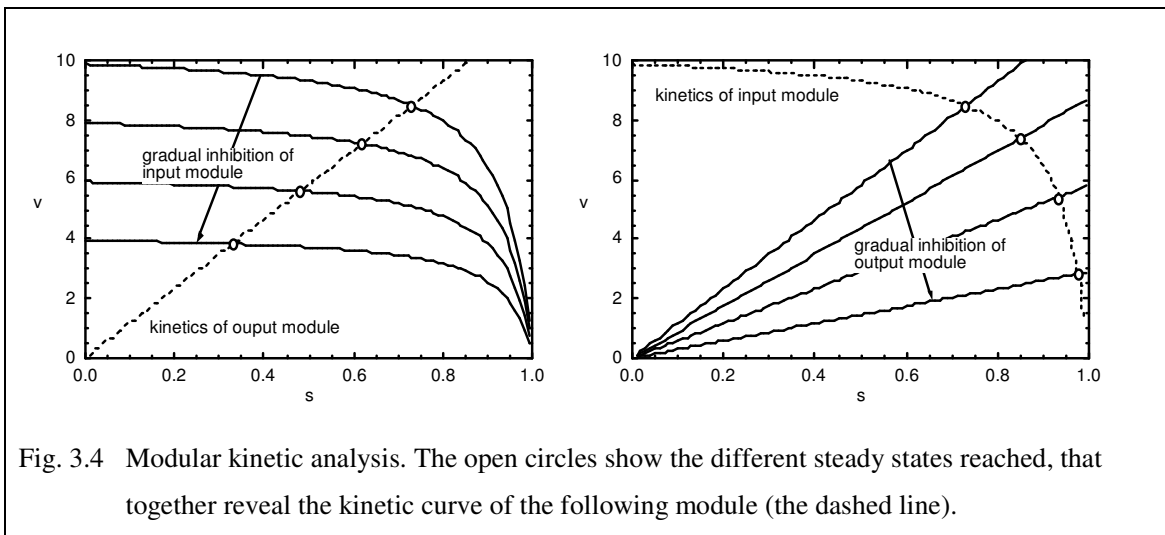


Fig. 3.4 Modular kinetic analysis. The open circles show the different steady states reached, that together reveal the kinetic curve of the following module (the dashed line).

Modular kinetic analysis is not limited to systems with just a single input and a single output. Depending on the possibilities to measure rates of different steps, application to more complex systems is possible.

Once we know the kinetic properties of two competing enzymes with respect to a common intermediate, we can predict how the distribution of flux between the two enzymes will be. In other words, the knowledge about the kinetic properties obtained by modular kinetic analysis leads to understanding of the flux distribution within a branched system such as the respiratory network.

### § 3.3 Choice of modules

When applying modular kinetic analysis to the respiratory network one has to decide which intermediate compound will be the center of attention. Studying phosphorylation by mitochondria from different sources, Brand and co-workers [1] have chosen  $\Delta\bar{\mu}_{H^+}$  as intermediate, with respiratory electron transfer as a whole as input module, and phosphorylation of ADP and membrane leak of  $H^+$  as two output modules. Van den Bergen et al. [2] have focused on Q as intermediate, and in plant mitochondria studied a system with two input modules (sdh and endh, see Fig. 3.2) and two output modules (aox and the complete set  $bc_1$ , cyt c, aa<sub>3</sub>, in Fig. 3.2). These two examples illustrates that a module need not be identical to a single enzyme, but can e.g. be a complete respiratory chain. In many cases practical considerations (is it easy to measure?) determine the choice of intermediate compound. When studying respiratory electron flux, cyt c is an obvious choice, as *in situ* cyt c reduction can be measured with a spectrophotometer.

### § 3.4 What we can learn from kinetic modelling

Sometimes data points ( $s$ ,  $J$ ) obtained by modular kinetic analysis or other means do not give enough information to predict behaviour of a respiratory network. This may occur when these data points are scattered, when we need to extrapolate outside of the range where  $s$  and  $J$  are varied, or when we want to consider what happens to the system when amounts of components (enzymes or intermediate compounds) are different from our experimental conditions. In some of these cases we can use the experimental data to generate, test and anchor a kinetic curve generated by a kinetic model. Once we have such a model, we can indeed do predictions about variations we have not (yet) experimentally tested.

In the present discussion of respiratory chain behaviour we will model enzymes as reversible Michaelis-Menten kinetics. As we apply our modular kinetic analysis to a single intermediate compound at the time, the same holds for our models, so we just consider a single substrate  $S$ , and a single product  $P$  formed from it. An interesting consequence is that we then have a conserved moiety in our model; the total amount of  $S + P +$  bound forms of  $S$  or  $P$  (mostly neglected) =  $q$  (constant ; c.f. § 1.3).

Application of Eq. 1.6 together with  $[S] + [P] = q$  leads to kinetic expressions of the form:

$$v = \frac{\alpha s + \beta}{\gamma s + 1} \quad (\text{Eq. 3.2})$$

for every single enzyme in our model. In Eq. 3.2  $s$  is the concentration of  $S$ ,  $\alpha$ ,  $\beta$  and  $\gamma$  depend on  $K_S$ ,  $K_P$ ,  $V_S$ ,  $V_P$  and  $q$ . When  $v$  is plotted as a function of  $s$ , hyperbolic (both concave or convex are possible) or linear (when  $\gamma = 0$ ) lines are obtained (see Fig. 1.2 for examples). In most cases (but not all) hyperbolic lines fit the data points obtained by modular kinetic analysis. Determination of  $\alpha$ ,  $\beta$  and  $\gamma$  by a fit procedure then produces a representative curve which can (with caution!) be used for extrapolation, and prediction of the effects of variation of enzyme concentration. Prediction of the effects of changing the total amount  $q$  of intermediate compound requires more involved kinetic modelling.

### § 3.5 The hard edge of the model: assumptions

Both modular kinetic analysis and kinetic modelling do rely very heavily on assumptions. It is good practice to keep these assumptions in mind all the time, and to try to test them experimentally as much as possible. Generally this is boring work, but it is essential, as the usefulness of model and kinetic data absolutely requires that the assumed conditions apply. The assumptions in a sense form the "hard edge" of the world within which we can use the model and apply the method.

Now what are the assumptions that are essential for modular kinetic analysis? Mainly that the only interaction between modules are via competition for common intermediates, and that other outside factors affecting the modules (e.g. when dealing with respiratory electron flux,  $\Delta \bar{\mu}_{H^+}$ ) remain constant. This includes sources (NADH, succinate) and sinks (oxygen) at the boundary of the modular system.

Also modelling brings its own assumptions, and fortunately many of these are identical to the ones that have to be made for the kinetic analysis. A prime example is the constancy of outside factors that affect rates within the system.

When dealing with assumptions, the golden rule is: "measuring is knowing".

### § 3.6 Steady states and control parameters

Once we have chosen a modular system of input and output enzymes centered around a single intermediate compound, and have assured ourselves that the conditions that together provide the hard edge of the system have been met, we can model the kinetics of the individual enzymes. With the resulting kinetic expressions we can calculate a steady state in which  $s$  does not change, and consequently the rates of the enzyme-catalysed steps do not change. The steady state is calculated by putting the sum of input rates equal to the sum of output rates, and solving for  $s$ .

From the same kinetic expressions (Eq. 3.2) the elasticities of the enzymes with respect to the variable  $s$  can be calculated:

$$\epsilon_s^i = \frac{\frac{\partial v_i}{\partial s}}{\frac{v_i}{s}} = \frac{s(\alpha_i - \beta_i \gamma_i)}{(\alpha_i s + \beta_i)(\gamma_i s + 1)} \quad (\text{Eq. 3.3})$$

So from  $s$ , all steady-state values of the  $J_i$  (Eq. 3.2) and  $\epsilon_s^i$  (Eq. 3.3) can be calculated.

Knowing these, the control coefficients can be calculated with the help of the theorems of metabolic control theory. For a system with  $n$  enzymatic steps, it is always possible to formulate  $n$  theorems for  $n$  system variables (leading to  $n^2$  theorems in total). For our system centered around a single intermediate, a set of  $n$  theorems for each of the system variables always include a summation theorem and a single connectivity theorem (there is only one intermediate). For the latter we need the  $\epsilon_s^i$ . When we have  $n$  enzymes all interacting with the intermediate,  $n - 2$  branching theorems can be formulated, which include the  $J_i$  in their expressions. The staple choices for system variables are the flux  $J$  and  $s$ . In the case of more inputs or outputs, e.g. the proportion of flux carried by the different branches can be used as variable.

Because this treatment yields  $n$  sets of  $n$  linear equations with  $n$  unknowns, things tend to become a little confused. A way to limit this confusion is to present these equations in matrix form [3] (which helps people who can handle matrix equations, but adds to the confusion of those who cannot).

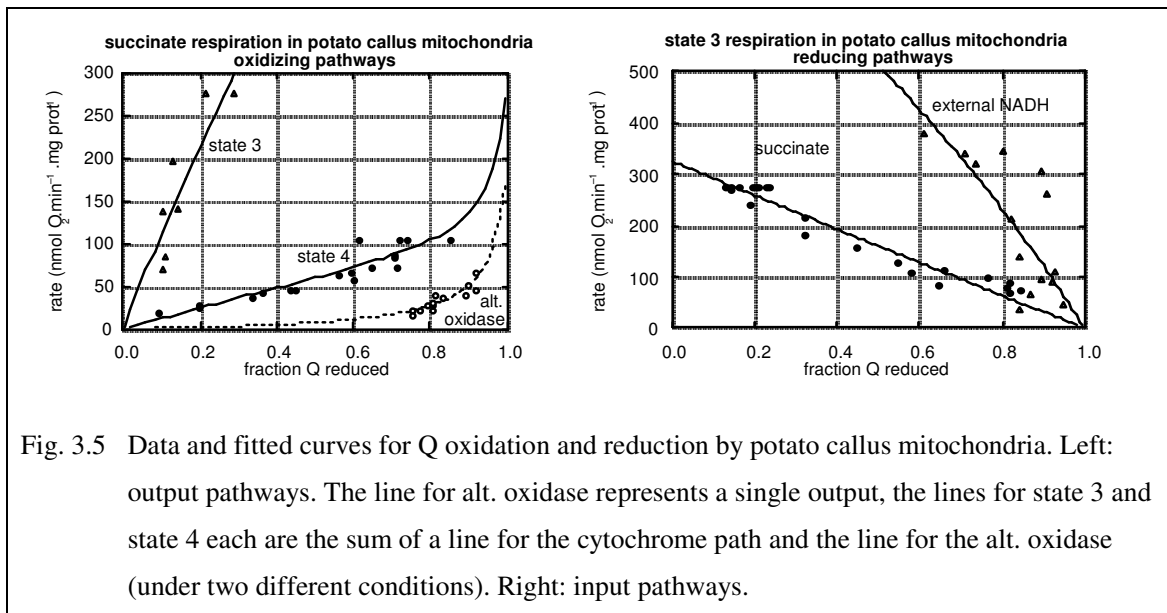


Fig. 3.5 Data and fitted curves for  $Q$  oxidation and reduction by potato callus mitochondria. Left: output pathways. The line for alt. oxidase represents a single output, the lines for state 3 and state 4 each are the sum of a line for the cytochrome path and the line for the alt. oxidase (under two different conditions). Right: input pathways.

### § 3.7 Example: Q branches in plant mitochondria and bacteria

The whole procedure has been applied to the branched respiratory network of potato callus mitochondria [2]. Q is chosen as central intermediate: two inputs have been studied, succinate dehydrogenase and external NADH dehydrogenase (sdh and endh, see Fig. 3.2) and in mitochondria from this plant tissue we find two oxidising pathways, the one containing all the cytochromes (called "cytochrome pathway", cyt) and the other the alternative oxidase (aox, see Fig. 3.2). Kinetic data have been gathered for these four components; for the two oxidising pathways (cyt, aox) by titration of sdh with the inhibitor malonate, for the two reducing pathways (sdh, endh) by titration of cyt with the inhibitor myxothiazol. Unwanted pathways were eliminated by leaving out the substrate (selection of input) or an overdose of appropriate inhibitor (selection of output).

After obtaining the four kinetic curves (see Fig. 3.5), the four enzymes have been modelled using Eq. 3.2, and then the steady state was calculated for the whole system in the absence of inhibitors. Then finally, the control parameters for the steady state were calculated. Because this is a four-component system, two branching theorems had to be formulated (c.f. Intermezzo 3; input branching between sdh and endh, and output branching between cyt and aox). This way in total 16 control coefficients were obtained, for 4 system properties: total flux  $J$ , intermediate concentration  $s$  and flux ratio's  $\frac{J_{ndh}}{J}$  and  $\frac{J_{cyt}}{J}$ . Table 3.I shows the 4 MCA theorems relating to flux control.

Table 3.I Summation, connectivity and branching theorems for flux control in the four-enzyme respiratory network consisting of two Q reducers (endh and sdh) and two QH<sub>2</sub> oxidisers (cyt and aox). The branching theorems are derived from Eq. 2.10 (total flux  $J$  does not figure in the "stretches" made up from Q reducers or QH<sub>2</sub> oxidisers, respectively). The negative signs (absent in Eq. 2.10) are caused by the fact that one flux in a stretch is encountered in opposite direction.

Summation theorem	$C_{endh}^J + C_{sdh}^J + C_{cyt}^J + C_{aox}^J = 1$
Connectivity theorem	$\epsilon_s^{endh} C_{endh}^J + \epsilon_s^{sdh} C_{sdh}^J + \epsilon_s^{cyt} C_{cyt}^J + \epsilon_s^{aox} C_{aox}^J = 0$
Branching theorem sdh / endh	$\frac{C_{endh}^J}{J_{endh}} - \frac{C_{sdh}^J}{J_{sdh}} = 0$
Branching theorem cyt / aox	$\frac{C_{cyt}^J}{J_{cyt}} - \frac{C_{aox}^J}{J_{aox}} = 0$

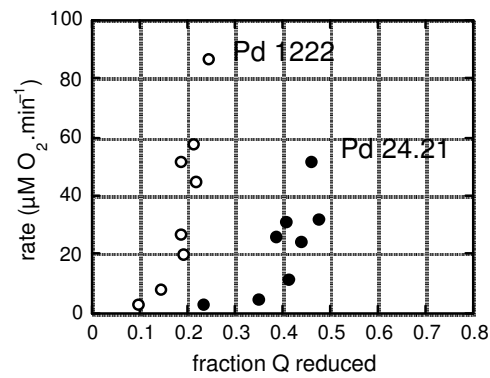


Fig. 3.6 Data for Q oxidation in respiratory membranes from two mutants of *P. denitrificans*.

Pd 1222 has the full complement of oxidising pathways, Pd 24.21 lacks the cytochrome  $bc_1$  complex and just has cytochrome  $ba_3$  (see Fig. 3.2).

The kinetics of the output pathways in respiratory membranes from aerobically grown *P. denitrificans* have also been characterised, yielding the result shown in Fig. 3.6. However, in this system a simple reversible Michaelis-Menten scheme is not sufficient to model the data satisfactorily.

### § 3.8 Dealing with poison: modulators

With the complete description of the steady state at our disposal, we have the possibility to predict changes that occur when the composition of the system is changed. That essentially is the use of e.g. the control coefficients. Another type of change that can be predicted is the effect of changes in kinetic parameters of the individual enzymes. This on the one hand provides a way to "soften up" the edge of the system, and on the other hand makes it possible to quantitatively predict the effects of other external factors on the steady state. An example of the first case is hidden in Fig. 3.5, where state 3 and state 4 differ in the value of  $\Delta\bar{\mu}_{H^+}$ . This value is assumed to be constant for a steady state, and for the *whole set of experiments* that have to be done to determine the kinetic curves by modular analysis. Nevertheless, by carrying out this procedure for two different conditions (in terms of  $\Delta\bar{\mu}_{H^+}$ ) we notice that only the shape of the cyt kinetic curve changes, and we get a good idea about how changes in  $\Delta\bar{\mu}_{H^+}$  may affect the steady state of the system. An example of the second case is quantification of the effect of modulators such as poisonous compounds on the system. One then has to perform the analysis (all the way, from determination of the kinetic curves to calculation of the steady state) in the absence and presence of (a few concentrations of) the modulator. Analysis of the different steady states immediately reveals how the modulator changes steady state values of  $s$ ,  $J$ , the  $\mathcal{E}_s^j$  and the control coefficients. It then is possible to understand effects of the modulator on e.g. the overall flux in terms of effects on the different individual steps in the system.

### § 3.9 Example: flavouring potatoes with cadmium salt

Kessler and Brand [4] have extensively studied the effects of Cd on respiratory phosphorylation in potato mitochondria. As the connecting intermediate in their analysis was

$\Delta \bar{\mu}_{H^+}$ , their input module was respiratory electron transfer and their output modules were phosphorylation of ADP and proton leak across the membrane (see Fig. 3.7). All three processes obviously are seen as movers of protons across the inner mitochondrial membrane. Their study included extensive testing of the hard edge of the system. By looking at the changes inflicted on control parameters they discovered that Cd had a twofold effect: respiration was inhibited, and proton leak was stimulated by the poison. The result was that phosphorylation (production of ATP) was decreased.

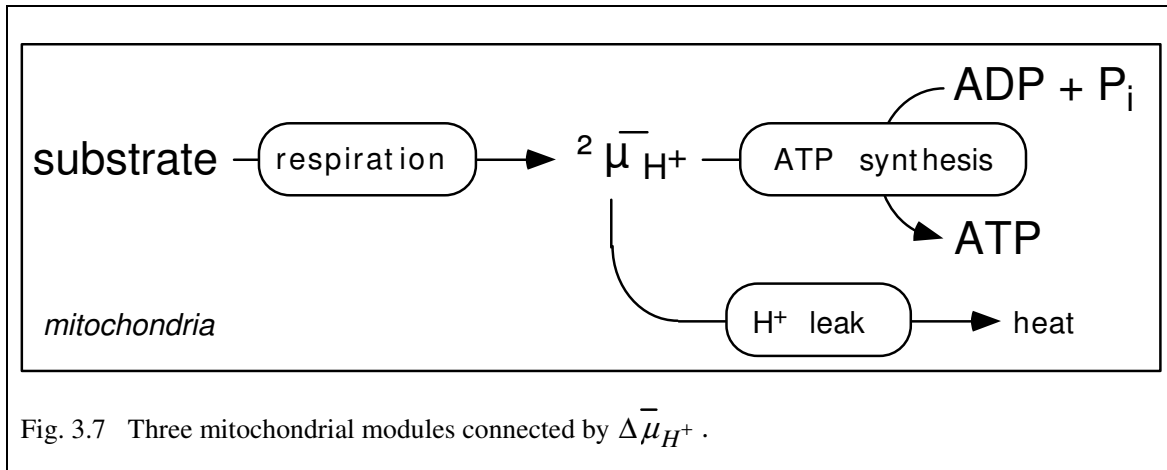


Fig. 3.7 Three mitochondrial modules connected by  $\Delta \bar{\mu}_{H^+}$ .

**Literature**

- [1] Hafner, R.P., Brown, G.C. and Brand, M.D. (1990) *Eur. J. Biochem.* 188, 313-319. Analysis of the control of respiration rate, phosphorylation rate, proton leak rate and proton motive force in isolated mitochondria using the 'top-down' approach of metabolic control theory.
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